Molecular detection and characterisation of potentially zoonotic bacteria in bathyergids from the Western Cape Province

By

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General abstract

Globally emphasis has been given to identify emerging and re-emerging pathogens. Rapid urban expansion creates a problem which is two-fold. Firstly, increasing slum living conditions due to inadequate rate of infrastructure development results in an increased reliance on natural resources, including the capture and consumption of surrounding wildlife to subsist, thereby facilitating the transfer of emergent zoonotic pathogens. Secondly, through activities such as pollution or alien species introductions, the rapid transformation of once pristine environments, alters natural systems, potentially exposing these environments to new bacterial pathogens. Therefore, the main aim of this study was to assess overlooked bacterial species harboured by four host species (*Bathyergus suillus, Georychus capensis, Cryptomys hottentotus hottentotus* and *Fukomys damarensis*) belonging to the subterranean rodent family Bathyergidae, which inhabit an environment well-suited for an array of bacterial species, and which varied in their exposure to human settlements. Bacterial prevalence and diversity was initially evaluated using broad-range PCR techniques in combination with nucleotide sequencing. This revealed high levels of bacterial prevalence (82.91%) and mixed infections (22.60%) in bathyergid species. Two bacterial groups, the *Bacillus cereus* complex (a group of soil-dwelling bacterial strains with pathogenic potential with an overall prevalence of 8.55%) and haemotropic *Mycoplasma* strains (vector-borne bacterial strains of zoonotic potential with an overall prevalence of 1.28%) were subsequently selected for further genetic analysis with genus and species-specific PCRs. *Bacillus* molecular screening and phylogenetic analyses was achieved by targeting four gene regions with seven published primer assays and two novel PCR assays. This enabled identification of two *B. cereus* complex strains in bathyergid lungs and revealed an overall *B. cereus* complex prevalence of 17.95% for the 234 bathyergid lung samples screened. *Bacillus* genome prevalence was significantly higher in *B. suillus* individuals (45.35%), sampled in a peri-urban environment, compared to the other bathyergid species sampled from pristine habitats (ranging from 0% - 4.44%). Anthropogenic activities in the area where *B. suillus* was sampled could, at least partially, attribute to the perceived difference between urban and naturally sampled bathyergid species, highlighting the role of *B. suillus* to act as both a reservoir of potentially zoonotic pathogens and as a sentinel for anthropogenic soiling. *Mycoplasma* molecular screening using three different PCR assays, all targeting the 16S rRNA gene region, confirmed an overall haemotropic *Mycoplasma* prevalence of 24.13% in the 286 bathyergid organs (lung, spleen and liver) screened. A significantly higher prevalence and diversity of haemotropic *Mycoplasma* strains was found in *B. suillus* lungs (41.86%) compared to its naturally occurring relatives (ranging from 0%-36%). Phylogenetic

analyses identified six novel haemotropic *Mycoplasma* strains, all grouping within a discrete monophyletic cluster, sister to *Mycoplasma coccoides*, and comprising two well-supported sub-clusters. The human introduction of commensal rodents harbouring *Mycoplasma* strains transferred through cosmopolitan arthropod vectors to indigenous bathyergids, likely underlies the higher prevalence in urban areas, although other biotic and abiotic factors affecting ectoparasite load also merit consideration. The data generated by the current study indicate the need to identify largely overlooked and potentially zoonotic bacterial pathogens in subterranean mammals and emphasises the importance of monitoring anthropogenically-introduced, opportunistic pathogens and the threats they pose to vulnerable communities and co-occurring, free-living animal species.

Key words: South Africa, indigenous rodents, bathyergids, anthropogenic activities, *Bacillus cereus* complex, haemotropic *Mycoplasma*, soil-borne pathogens, vector-borne pathogens

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Declaration

I, Liezl Retief, declare that the thesis/dissertation, which I hereby submit for the degree Magister Scientiae (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.

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Disclaimer

This dissertation contains two research chapters which have been prepared as stand-alone manuscripts for publication purposes. Therefore, some unavoidable repetition may occur between chapters.

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Chapter 1

General introduction

1.1. Background

It is estimated that globally, more than half of emerging and re-emerging infectious diseases are caused by bacterial species (Jones et al. 2008). These infectious diseases continue to cause high rates of mortality and, with the continual encroachment of human settlements on natural habitats the risk of contact with wildlife harbouring emerging and re-emerging disease pathogens has increased (Comer 2001; Yildiz 2007). Consequently, the threat of zoonotic pathogen transfer in both rural and urban communities has amplified dramatically (Comer 2001; McMichael 2004). Moreover, although bacterial zoonotic diseases have been implicated in vast socio-economic losses worldwide, many questions about the biology of these bacterial pathogens outside their human hosts are still largely unanswered (Cox 1979; Yildiz 2007; Meerburg 2009; Alemayehu 2012). Understanding where these zoonotic pathogens are emerging from can aid in developing protocols to prevent the spread of these diseases to humans (Blancou et al. 2005). For instance, in 1887 Captain David Bruce was able to isolate *Brucella melitensis*, a bacterium which is the causative agent of Malta fever in humans, from goats, thereby identifying them as the natural reservoir of this pathogen (Corbel 1997; Nicoletti 2002; Blancou et al. 2005). This enabled the development of protocols, such as the prohibition of drinking unpasteurised goat milk to limit the further zoonotic transfer of this pathogen between goats and humans (Nicoletti 2002; Blancou et al. 2005).

1.1.1. Emerging bacterial pathogens in South Africa

Africa is the continent with the second largest expansion rate of urban areas worldwide (De Vries et al. 2014). This rapid urbanisation results in a significant increase in slum living conditions and, due to improper waste disposal within these areas, animals, such as rats and other rodents, which are potential carriers of pathogenic soil-borne as well as vector-borne bacteria, are attracted by this new food source to informal settlements (Gratz 1999; Ostfeld 2009; Benacer et al. 2013; De Vries et al. 2014). This increases the risk of human-animal interactions facilitating the transmission of opportunistic bacterial pathogens (Gratz 1999; Ostfeld 2009; Benacer et al. 2013; De Vries et al. 2014). Furthermore, these informal settlements often have poor infrastructure in

place, resulting in people being more heavily reliant on natural bodies of water for sanitation, soil systems for small-scale agriculture, and wildlife as alternative sources of protein, all of which are potential sources of opportunistic infections (Duran-Alvarez & Jimenez-Cisneros 2014).

In the Western Cape Province of South Africa concern has been raised about the potentially pathogenic bacterial genera present within the natural water systems (Jackson et al. 2009; Paulse et al. 2012). Paulse et al (2012) investigated the bacterial community structure present in the Berg and Plankenburg rivers through universal 16S bacterial screening. They isolated a number of opportunistic pathogenic bacterial species including *Pseudomonas aeruginosa*, *Staphylococcus* sp., and *Bacillus cereu*s as well as *Aeromonas* sp., *Acinetobacter* sp., *Stenotrophomonas* sp. and *Yersinia enterocolitica* from both the Berg and Plankenburg rivers. These water systems serve as water reservoirs for various industrial, agricultural, domestic and municipal activities and, as one of the major avenues of soil pollution is contamination by waste water, the surrounding soil systems likely also harbour these opportunistic pathogens (Jackson et al. 2009; Duran-Alvarez & Jimenez-Cisneros 2014).

Due to limited resources, poorer communities living within the Western Cape Province of South Africa rely on alternative protein sources including the Cape dune mole-rat (*Bathyergus suillus*) (De Graaff 1981). The capture and butchering of these animals creates a possible route for the transfer of both soil and blood-borne pathogens, either through direct contact or via blood-sucking arthropods associated with these mole-rats. This is of particular concern as up to 30% of people living in and around Cape Town are HIV-positive, making them more susceptible to zoonotic pathogens (Comer 2001; Kenyon et al. 2013).

1.1.2. Rodents as reservoirs of disease

Rodent species throughout the world have been implicated in the spread of various bacterial, viral, protozoan and fungal diseases (Cox 1979). There are various routes by which rodents can either directly or indirectly transmit diseases to humans (Meerburg 2009). Direct transmission routes include cases where pathogens are transmitted through a rodent bite or scratch to humans, or cases where humans are exposed to contaminants released from rodent faecal matter and urine, either by breathing in airborne contaminants or through drinking or eating of liquids and food contaminated by rodent excrement (McMichael 2004; Meerburg 2009). As ectoparasitic arthropods (such as ticks or fleas) feed both on rodents and humans, indirect transmission of

rodent-borne pathogens can occur where these arthropods act as vectors of infectious agents (Comer 2001). Furthermore, although not as common as other transmission routes, pathogens can also be transmitted to humans through poorly cooked meat from an animal that may have become infected through direct or indirect exposure to infected rodents (Meerburg 2009).

1.1.3. The family Bathyergidae

Bathyergus suillus (the Cape dune mole-rat) is the largest species belonging to the family Bathyergidae (African mole-rats) (De Graaff 1981). Members of the family Bathyergidae are subterranean mammals of the order Rodentia, consisting of around 24 species and six distinct genera (Hubálek et al. 2005). Of the six genera *Bathyergus*, *Georychus* and *Heliophobius* are solitary and only pair up during the mating season, or when young are reared by their mothers, while *Cryptomys*, *Fukomys* and *Heterocephalus* are social mammals (Sichilima et al. 2008; Lövy et al. 2012). In the Western Cape Province, the solitary species *B. suillus* occurs sympatrically with two other bathyergid species, namely the Common mole-rat (*Cryptomys hottentotus hottentotus*) and the Cape mole-rat (*Georychus capensis*) (De Graaff 1981).

Cryptomys hottentotus hottentotus prefers semi-arid habitats, whereas both *B. suillus* and *G. capensis* are restricted to areas with higher rainfall (Davies & Jarvis 1986). Due to the wide distribution of both *C. h. hottentotus* and, to a certain extent, *G. capensis* (Table 1.1), these species overlap in distribution with other members of the bathyergid family (Skinner & Smithers 1990). *Fukomys damarensis* (the Damaraland mole-rat) has an overlapping distribution with *C. h. hottentotus* (Bennett 2011; Lövy et al. 2012). These four bathyergid species vary in the degree of sociality, above ground activity, body mass, burrowing behaviour as well as soil preference (summarised in Table 1.1).

Table 1.1: Weight, geographical distribution, level of sociality, above ground activity and soil preference of the four bathyergid species (*Bathyergus suillus, Crypromys hottentotus hottentotus, Georychus capensis* and *Fukomys damarensis*) evaluated in this study for the presence of potentially zoonotic species of the *Bacillus* and *Mycoplasma* bacterial genera.

Species	Average weight	Distribution	**Level of sociality**	**Evidence of above ground activity**	**Soil preference**
B. suillus	504-1291 g	Endemic to Cape floristic region	Solitary	Yes	Sandy soils
C. h. hottentotus	27-84 g	Restricted to South Africa	Social	Yes	Loam/clay soils
G. capensis	44-287 g	South Africa, Namibia, Botswana and Zimbabwe	Solitary	Yes	Loam/clay soils
F. damarensis	131 g	Endemic to the western parts of southern Africa (including parts of South Africa and Zambia)	Eusocial	Rare	Loose, arid soils

Sources: De Graaff 1981; Skinner & Smithers 1990; Jarvis & Bennett 1993; Bennett 2011; Robb et al. 2012; Lövy et al. 2012

Although these mole-rat species have a partially overlapping distribution, *B. suillus* prefers sandy soil that is less compact compared to the loam clay soils preferred by *C. h. hottentotus* and *G. capensis* (De Graaff 1981; Robb et al. 2012). This is because *B. suillus*, being relatively large in size, requires softer soils to be able to dig and be truly subterranean (Davies & Jarvis 1986). These solitary mole-rats dig extensive tunnels with both shallow (between 40 and 65 cm in depth) and deeper tunnels (more than 2 m in depth) present in their burrow system (Davies & Jarvis 1986; Thomas et al. 2009). *Bathyergus suillus* occurs in abundance in farmlands and in more urbanised areas and, although *B. suillus* does not often change the geometry of their burrows, they do back-fill older, unused parts of their burrow system which they may re-excavate at a later stage (Davies & Jarvis 1986; Kotze et al. 2006). These authors also suggest that *B. suillus* may change their nesting sites frequently within their burrow system to minimise the effect of parasites. *Bathyergus suillus* individuals are not only sexually dimorphic in size, with males being larger, but males generally also have longer burrow systems compared to females (Thomas et al. 2009). Unlike other bathyergids, *Bathyergus suillus* is often found walking on the surface during daytime (Skinner & Smithers 1990).

Cryptomys hottentotus hottentotus construct some of the longest burrows of all bathyergids, when the size of the animal is taken into consideration (Davies & Jarvis 1986). This social species constructs elaborate burrow systems with many side branches and lives in colonies of up to 14 individuals (De Graaff 1981; Skinner & Smithers 1990). There appears to be some

variation in burrowing behaviour and dispersal activities between arid and mesic populations, with arid populations having shallower tunnels and dispersing less often than mesic populations (Spinks et al. 2000). These mole-rats prefer semi-arid habitats and some evidence exists that they come above ground at night, as their remains have been found in owl scats (Bennett 1989; Skinner & Smithers 1990).

Less is known about the habits of *G. capensis* compared to *B. suillus* and *C. h. hottentotus* (De Graaff 1981). Although these mole-rats burrow shallowly (tunnel depths between three and nine centimetres in depth) and form small mounds, they do have extensive burrow systems, with many side tunnels (De Graaff 1981; Du Toit et al. 1985; Skinner & Smithers 1990; Roper et al. 2001). Juveniles either disperse from the burrows where they are born by extending the burrows of their mothers and later sealing themselves off from the parental burrow system, or they can disperse by moving above ground (Bennett & Jarvis 1988).

Fukomys damarensis is a xerophilic mole-rat species that prefers loose, sandy soils, occurring in areas that have low, unpredictable rainfall (Bennett 2011; Lövy et al. 2012). This mole-rat species is eusocial, consisting of one breeding female and, typically, one breeding male along with their non-breeding offspring (Jarvis et al. 1998). Generally, *F. damarensis* colonies consist of around 12 individuals, but colonies can vary between two and 40 individuals (Bennett 2011). *Fukomys damarensis* dig extensive burrows, typically consisting of unbranching, tunnelling systems which extend outward from a deep nest (usually more than 2.4m below the ground) (Jarvis et al. 1998; Bennett 2011).

As a result of their subterranean lifestyle, these four bathyergid species live in a microclimate with warm, moist air, low in oxygen; an environment well suited to an array of microbes that may hold pathogenic potential (Reichman & Smith 1990; Roper et al. 2001). Furthermore, given that relatively few studies have focused on potential pathogens within indigenous rodent communities in South Africa (Taylor et al. 2008), that people dwelling in rural settlements have increased susceptibility to contagious diseases (Kenyon et al. 2013) and that animal handling facilitates zoonotic transfer between *B. suillus* and humans, investigating the potential of *B. suillus* and its relatives to be host to and act as sentinels for potentially pathogenic soil bacteria, is warranted.

1.1.4. Justification

In a preliminary study done on the bacterial prevalence in the lungs of *B. suillus, C. h. hottentotus, G. capensis* and *F. damarensis*, PCR amplification with universal 16S rRNA bacterial primers revealed that 194 of the 234 lung samples screened were positive for bacterial genome presence (Retief 2014). Of these, 22.60% produced mixed sequences, most likely due to the presence of more than one bacterial pathogen within the same sample. Nucleotide BLAST searches against the GenBank database (www.ncbi.nlm.nih.giv/blastn) revealed high levels of bacterial diversity with a total of 13 genera being identified. Many of the bacterial genera contained species of zoonotic potential and were medically significant (Table 1.2). Bacterial strains belonging to the *Bacillus cereus* complex were most prevalent in *B. suillus* (Table 1.2; Figure 1.1). This bacterial group consists of seven recognised species that have varying levels of pathogenicity (Guinebretière et al. 2013). Nucleotide blastn searches and phylogenetic analyses conducted in 2014 (Retief 2014) also revealed a relatively high prevalence of haemotropic *Mycoplasma* strains in different bathyergid species (Table 1.2; Figure 1.1). Haemotropic *Mycoplasma* strains have been implicated in causing haemolytic anaemia in various hosts (Messick 2004) and have previously been linked to mortalities in captive mole-rat colonies (unpublished results, Bastos, Lutermann & Bennett 2013). Therefore, considering that both *Bacillus cereus* complex strains, as well as haemotropic *Mycoplasma* strains, have previously been found to infect bathyergids that occur in the south-western part of South Africa, further investigation into the dynamics of these potential pathogens is important.

Table 1.2: Summary of bacterial genera found to be present in the lungs of four bathyergid species, ranked by health risk posed to humans and zoonotic potential (from Retief, 2014).

Bacterial genus	Overall Prevalence	Host species	Prevalence per host species	Health risk	Zoonotic concern
Bacillus sp.	8.55%	*B. suillus*	23.26%	Very high	Yes
Staphylococcus sp.	4.70%	*B. suillus*	3.49%	Very high	Yes
		F. damarensis	12%		
		C. h hottentotus	4.44%		
Mycoplasma sp.	1.28%	*B. suillus*	1.16%	High	Yes
		F. damarensis	4%		
Clostridium sp.	1.71%	*F. damarensis*	8%	High	Yes
Bartonella sp.	1.28%	*B. suillus*	2.33%	High	Yes
		C. h hottentotus	2.22%		
Pseudomonas sp.	2.56%	*B. suillus*	4.65%	Moderate	Yes
		F. damarensis	4%		
Salmonella sp.	0.43%	*B. suillus*	1.16%	Moderate	Yes
Caulobacter sp.	3.85%	*B. suillus*	2.33%	Low	No
		C. h hottentotus	8%		
		G. capensis	5.66%		
Lactococcus sp.	1.28%	*B. suillus*	2.32%	Low	Possible
		F. damarensis	2%		
Sporosacina sp.	2.99%	*C. h hottentotus*	15.56%	Very low	No
Carnobacteria sp.	0.86%	*C. h hottentotus*	4.44%	Very low	No
Sphingomonas sp.	0.85%	*G. capensis*	3.77%	Very low	Unknown
Brochotrix sp.	0.85%	*C. h hottentotus*	4.44%	None known	No

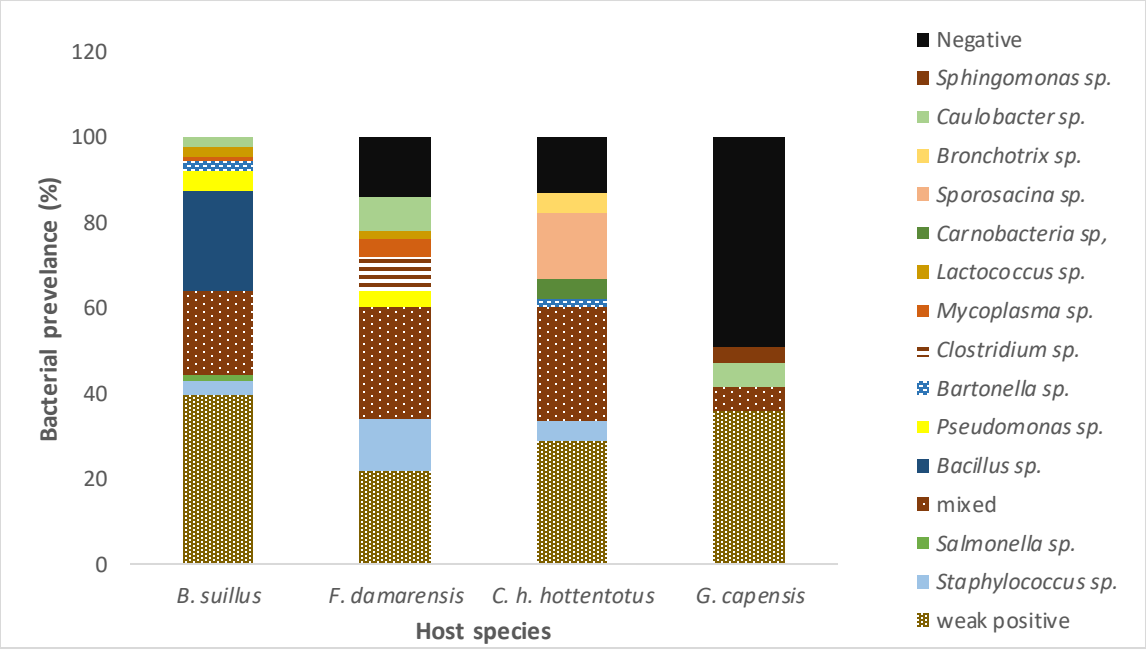

Figure 1.1: Percentage of different bacterial genera found in the lung tissues of four bathyergid species (taken from Retief 2014). Bathyergid species are indicated below the bars (*B. suillus* = *Bathyergus suillus*, *F. damarensis* = *Fukomys damarensis*, *C. h. hottentotus* = *Cryptomys hottentotus hottentotus* and *G. capensis*=*Georychus capensis*). A significant difference in overall bacterial prevalence was found between the four different bathyergid species.

1.2. Soil-borne pathogens

1.2.1. Host and non-host environment

Microorganisms are significantly influenced by their environment (Wilson & Slayers 2003). Traditionally the environment of pathogens has been defined as the hosts which they infect and as a result most research has focused on host-pathogen interactions (Soborg et al. 2013). However, in recent years it has become apparent that the transmission and maintenance of potential pathogens is not only dependent on their ability to survive in different hosts, but also on their ability to survive and propagate in environments outside the host (Yildiz 2007). Consequently, understanding how pathogens behave in and are influenced by both the host and the non-host environment is critical (Wilson & Slayers 2003; Yildiz 2007; Soborg et al. 2013).

1.2.2. The rhizosphere

With the recent surge in opportunistic bacterial pathogens in immune-compromised patients, made more susceptible due to diseases such as HIV, cystic fibrosis or healthcare-associated infections, greater focus on both the ecology and pathogenesis of these re-emergent and newly emergent pathogens, is required (Berg et al. 2005; Aujoulat et al. 2012). However, research on these aspects, is limited.

Many emergent pathogens belong to genera which are soil-associated, often forming bivalent interactions, growing both in the rhizosphere, the microenvironment which connects soil and plant roots, as well as opportunistically infecting animal hosts (Berg et al. 2005; Aujoulat et al. 2012). Bacterial genera falling into this category include, among others, *Burkholderia, Enterobacter, Herbaspirillum, Ochrobactrum, Pseudomonas, Ralstonia, Staphylococcus* and *Stenotrophonomans* (Berg et al. 2005; Aujolat et al. 2012). As a result, it has been postulated that the rhizosphere may be a "hotspot" for the evolution of opportunistic bacterial pathogens (Berg et al. 2005; Aujoulat et al. 2012; Baumgardner 2012).

The rationale for this suggestion of pathogen evolution in a non-host environment is as follows. The rhizosphere is a habitat greatly influenced by root metabolism (Berg et al. 2005). Due to exudation of compounds such as organic acids, sugars and vitamins from roots, along with other forms of rhizodeposition of plants, such as sloughing-off of root cap cells, the rhizosphere is a nutrient-rich niche capable of supporting a wide array of bacterial communities (Berg et al. 2005; Aujoulat et al. 2012; Baumgardner 2012). Within this high-nutrient environment; protozoal, fungal

and bacterial communities face high levels of competition and, as a result, many soil-dwelling microbes have evolved to excrete antibiotic substances, in an attempt to competitively exclude bacterial strains. This, in turn, leads to the selection for bacteria with multiple antibiotic-resistances within this microenvironment (Berg et al. 2005). Moreover, the intense competition between soil microbes can lead to the selection of bacterial strains with antagonistic actions toward eukaryote cells (Berg et al. 2005; Baumgardner 2012). These selection processes can then predispose bacterial species to become pathogenic should they come into contact with humans or other animals.

Importantly, the high nutrient content of the rhizosphere microenvironment also facilitates the exchange of genetic material between different bacterial species or even different bacterial genera (Berg et al. 2005; Duran-Alvarez & Jimenez-Cisneros 2014). This is known as horizontal gene transfer (HGT) and represents a major avenue for the rapid evolution of different bacterial species (Hacker & Carniel 2001; Baumgardner 2012). The genomes of prokaryotes consist of a core gene pool, which includes genes encoding proteins essential for housekeeping functions and which is generally not transferred between different prokaryotes, as well as a flexible gene pool, which is not essential for cellular function and can be exchanged between prokaryotes (Hacker & Carniel 2001). Gene regions encompassed by the flexible gene pool have been termed genomic islands and encode functions such as antibiotic-resistance, metabolic activities and pathogenesis (Hacker & Kaper 2000). Therefore, genomic islands have the potential to increase fitness in microbes depending on the environment they inhabit at the time (Hacker et al. 2003). Furthermore, genomic islands may confer a fitness advantage to their bacterial carriers in more than one environment (Hacker & Carniel 2001). For example, the iron-uptake system, known as yersiniabactin is encoded by genes found in highly pathogenic strains belonging to the genus *Yersinia*, but the same genes have also been identified in soil-dwelling *Klebsiella* strains, as well as in *E. coli* strains which form part of the harmless intestinal flora in humans, predisposing these bacterial strains to survive in diverse iron-limiting environments (Hacker & Carniel 2001; Hacker et al. 2003). As a result, it is clear that certain genes can be advantageous in different environments and that HGT can result in the rapid evolution and specific selection of certain traits that could predispose soil-dwelling bacteria to become opportunistic pathogens, thereby emphasising the need to better understand the dynamics of soil-associated bacteria.

1.2.3. Influence of human-transformation

Bacterial colonisation of both the rhizosphere and other portions of the soil environment is strongly influenced by the structure, temperature, pH, nutrient-type, plant species presence and moisture in the soil (Berg et al. 2005; Baumgardner 2012). Opportunistic pathogens can occur naturally in soil, be introduced intentionally, through practices such as bioremediation or biocontrol, or accidentally, through anthropogenic activities resulting in pollution of soil systems (Baumgardner 2012; Duran-Alvarez & Jimenez-Cisneros 2014). Of growing concern is that waste water, released into the environment, may be supplying a constant influx of both antibiotic-resistant pathogens as well as antibiotic-resistant genes to different soil systems, which could potentially be transferred to other soil bacteria through HGT (Duran-Alvarez & Jimenez-Cisneros 2014).

Due to ever increasing encroachment of humans into natural areas, especially in developing countries such as South Africa, not only is the risk of introducing novel bacteria into naïve environments amplified, but there is an increased risk of humans coming into contact with these and resident soil pathogens, to which they have previously had limited exposure. This contact can be direct through activities, such as traditional farming or other agricultural practices, resulting in humans coming into contact with soil or water harbouring opportunistic bacterial pathogens (Duran-Alvarez & Jimenez-Cisneros 2014). Contact can also be indirect where humans can become infected after coming into contact with animals or plants carrying these opportunistic bacterial pathogens (Duran-Alvarez & Jimenez-Cisneros 2014).

1.2.4. The *Bacillus cereus* complex

1.2.4.1. Species

The *Bacillus cereus* complex consists of gram-positive, spore-forming bacterial species which form part of the *Bacillus* genus (Jensen et al. 2003; Thorsen et al. 2006; Tourasse et al. 2006). *Bacillus cereus* complex species are most commonly isolated from different soil environments and to date, nine species with varying degrees of medical and economic importance have been formally recognised (Tourasse et al. 2006, 2011). The nine recognised species, which share a high degree of genetic similarity, include: *B. cereus, B. anthracis, B. thuringiensis, B. bombysepticus, B. cytotoxicus, B. toyonensis, B. weihenstephanensis, B. mycoides* and *B.*

pseudomycoides (Jensen et al. 2003; Thorsen et al. 2006; Tourasse et al. 2006; Guinebretière et al. 2013, Jiménez et al. 2013; Cheng et al. 2014).

Bacillus cereus strains are ubiquitous in soil environments resulting in food-borne infections worldwide (Kotiranta et al. 2000). Due to their ability to survive food preservation methods such as pasteurisation and heating, these motile bacteria can cause two clinically distinct forms of food poisoning: diarrhoeal (which is characterised by abdominal pain and diarrhoea) and emetic (which is characterised by nausea and vomiting) (Kotiranta et al. 2000; Jensen et al. 2003). Diarrhoeal food poisoning is caused by enterotoxins, one haemolytic and the other non-haemolytic, during vegetative growth while emetic food poisoning is caused by a toxin called cereulide produced during cellular growth in food (Granum & Lund 1997). Strains of *B. cereus* are also associated with causing septicaemia, endophthalmitis, as well as pneumonia in immune-compromised patients and in the USA, have been known to cause fatalities in immune-competent patients (Hoffmaster et al. 2006).

Bacillus anthracis infects a wide range of mammalian hosts, including humans, and is considered an obligate mammalian pathogen (Tourasse et al. 2006). This non-motile bacterium is the causative agent of anthrax, an often fatal disease with symptoms similar to haemorrhagic fevers and a history of being used as a bioterrorism agent (Hendriksen et al. 2006; Fasanella et al. 2010). *Bacillus anthracis* strains are non-haemolytic and possess two plasmid genes, pXO1 and pXO2, which are responsible for the virulence of this pathogen (Jensen et al. 2003). Relatively uncommon around the world since the development of vaccines, *B. anthracis* is still rampant in parts of Africa, with infected animals often being reported in national game parks such as the Kgalagadi Transfrontier Park, as well as the Vaalbos and the Kruger National Parks in southern Africa (Fasanella et al. 2010). The infection has traditionally been considered not to be contagious, but it has been reported that feeding on a freshly killed carcass of an infected animal can result in infection if sufficiently high levels of *B. anthracis* endospores are ingested (Beyer & Turnbull 2009). Although *B. anthracis* has been known to cause disease in both humans and animals since ancient times, it is still unclear how the disease is contracted under natural conditions or how outbreaks occur (Beyer & Turnbull 2009).

Bacillus thuringiensis can only be distinguished from *B. cereus* by the insecticidal parasporal crystalline proteins it produces during sporulation which contain δ-endotoxins encoded by genes located on transportable plasmids (Jensen et al. 2003; Hendriksen et al. 2006). As a result,

this bacterial species has found worldwide application as a microbiological control agent for insect larvae (Hendriksen et al. 2006; Bishop et al. 2014). Although mostly associated with causing pathogenicity in invertebrates, there is evidence of this species causing infections in humans (Tourasse et al. 2006). *Bacillus bombysepticus* is closely related to *B. thuringiensis* and affects silkworm larvae by producing parasporal crystals which are highly toxic to silkworm larvae (Cheng et al. 2014).

Less is known about the remaining species within the *B. cereus* complex (Ahmod et al. 2011). It is generally considered that *B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* are non-pathogenic (Ahmod et al. 2011). *Bacillus weihenstephanensis* possesses cold-shock protein genes and is a psycho-tolerant species (Tourasse et al. 2006). *Bacillus mycoides* and *B. pseudomycoides* have been found in a range of environments and are capable of rhizoid growth. It is believed that these two species may have an antagonistic effect on certain fungal species (Jensen et al. 2003). *Bacillus mycoides* and *B. weihenstephanensis* are considered by some to be ecotypes of one another and it has been suggested that these two species should not be classified as separate species (Tourasse et al. 2006). Although strains belonging to both ecotypes are generally considered to be non-pathogenic, a number of studies have found evidence to suggest that these species may possess genes capable of producing pathogenic factors similar to those produced by *B. cereus,* with some *B. mycoides* strains showing sequence identities of 99.5% to that of *B. cereus* (Stenfors et al. 2002; Bavykin et al. 2004; Hendriksen et al. 2006; Thorsen et al. 2006).

Bacillus toyonensis is closely related to *B. cereus,* and has only recently been given specific status, but has been used as a probiotic in animal nutrition since 1975 (then classified as a subspecies of *B. cereus*) and shown to prevent various gastrointestinal disorders (Jiménez et al. 2013). Recently another new species belonging to the *B. cereus* complex has been identified on the basis of 16S rRNA, multilocus sequence typing (MLST) and DNA:DNA hybridisation data (Guinebretière et al. 2013). This novel species, *B. cytotoxicus* is thermotolerant, growing at temperatures ranging from 20ºC to 50ºC (Guinebretière et al. 2013), and is associated with producing an enterotoxin, known as cytokine K, which results in severe diarrhoeic food-poisoning (Tourasse et al. 2011; Guinebretière et al. 2013).

1.2.4.2. Taxonomy

Various gene and genome studies have failed to definitively distinguish between the species belonging to the *B. cereus* complex, owing to the high levels of genetic similarities displayed between members within this phylogenetic cluster (Tourasse et al. 2006; Didelot et al. 2009). Classification into nine species has been based on differences in phenotypic, immunological and biochemical characteristics and was thus reliant on methods that are often tedious, expensive and generally inaccurate (Blackwood et al. 2004; Bavykin et al. 2004). As a result, the *B. cereus* complex has had a contentious taxonomic history (Didelot et al. 2009).

Some have argued that *B. cereus* complex species should be seen as a single species and support for this suggestion has come from different lines of evidence. Multilocus enzyme electrophoresis (MEE) has shown that, with regard to their chromosomal genes, *B. cereus* complex species share very high levels of genetic similarity and that distinguishing between different species is only possible by looking at plasmid gene regions, which can be exchanged from one species to another through HGT (Helgason et al. 2000). DNA:DNA hybridisation studies have also found a very high degree of homology between certain *B. cereus* complex species (Seki et al. 1978). Further support for the classification into a single species is that *B. cereus* species show very high levels of sequence conservation across the 16S rRNA gene region (Sacchi et al. 2002).

However, based on more recent studies using methods such as MLST and DNA:DNA hybridisation, some members of the *B. cereus* complex appear to constitute separate species. These phylogenetic studies have shown that the nine *B. cereus* complex species form seven discrete phylogenetic clusters, with strains of some *B. cereus* complex species occurring within multiple phylogenetic clusters (Table 1.3) (Tourasse et al. 2006; Didelot et al. 2009; Ceuppens et al. 2013). Species exempt from this shifting between different phylogenetic clusters include *Bacillus anthracis*, which is a highly clonal species and *B. pseudomycoides* as well as *B. cytotoxicus*, which form well-defined outgroups within the *B. cereus* complex (Tourasse et al. 2011). In contrast, *B. cereus, B. thuringiensis* as well as *B. mycoides* do not form discrete clusters, and therefore some have suggested that these three species may not constitute monophyletic species (Zwick et al. 2012).

Table 1.3: Seven phylogenetic clusters of *B. cereus* group species (adapted from Guinebretière et al. (2010)).

Phylogenetic group	*B. cereus* group species	Growing temperature range (ºC)
I	*B. pseudomycoides*	10 – 43
II	*B. cereus*	7 – 40
	B. thuringiensis	
III	*B. cereus*	15 – 45
	B. thuringiensis	
	B. anthracis	
IV	*B. cereus*	10 – 45
	B. thuringiensis	
V	*B. cereus*	8 – 40
	B. thuringiensis	
VI	*B. weihenstephanensis*	5 – 37
	B. mycoides	
	B. thuringiensis	
VII	*B. cytotoxicus*	20 – 50

1.2.4.3. Horizontal gene transfer (HGT)

Complicating phylogenetic analyses of the *B. cereus* complex further is the possibility that plasmid-encoded genes can be lost to the environment (Ceuppens et al. 2013). Furthermore, HGT of plasmid genes between different *B. cereus* complex species has also been suggested (Ceuppens et al. 2013). Evidence of HGT comes from MLST analyses as some of the gene regions used during these analyses show contrasting phylogenies (Tourasse et al. 2011). This is of particular importance considering that the toxins produced by various pathogenic *B. cereus* complex species are encoded by gene regions located on plasmids and that these toxin-encoding genes are often the only way to genetically distinguish between different *B. cereus* complex species (Rasko et al. 2005; Ceuppens et al. 2013). If HGT between *B. cereus* complex species exists, it is conceivable that non-pathogenic *B. cereus* complex species occupying the same ecological space (such as the rhizosphere), as their close pathogenic relatives can obtain plasmid-encoded virulence factors. It has, however, been suggested that additional, unknown genetic factors may be necessary for a *B. cereus* complex strain to become virulent, such as the mutual adaptation of both chromosomal and plasmid genes (Helgason et al. 2000; Jensen et al. 2003). All nine species do seem to have the potential to be pathogenic, as all possess chromosomal genes capable of encoding enterotoxins as well as digestive enzymes which can lead to gastrointestinal illnesses (Tourasse et al. 2011). Moreover, using next-generation sequencing technologies, Zwick et al. (2012), demonstrated that no *B. anthracis* strains or other *B. cereus* complex strains possessing the pOX1 plasmid seem to

have undergone selection pressure in their chromosomal genomes which could result in improved virulence. This has led to the suggestion that many of the *B. cereus* complex species may be capable of obtaining anthrax-like virulence through HGT (Mandic-Mulec et al. 2015).

It is known that HGT between *B. cereus* complex species occurs in the gut of both earthworms as well as lepidopteran larvae (Jensen et al. 2003). Furthermore, it has been shown experimentally that some *B. cereus* complex species, such as *B. cereus* and *B. mycoides,* are capable of forming multi-cellular communities within soil environments (Vilain et al. 2006). These communities resemble biofilms, but cannot be characterised as true biofilms as they do not occur on a surface, which is a defining characteristic of biofilms (Vilain et al. 2006; Yildiz 2007). Nevertheless, as biofilms can facilitate gene transfer between microorganisms, it has been suggested that this formation of multi-cellular communities may facilitate gene transfer between different *B. cereus* complex species (Wilson & Slayers 2003). This is of particular interest as *B. anthracis* is also capable of forming biofilms within the rhizosphere (Saile & Koehler 2006).

1.2.4.4. Hosts species

Bacillus cereus complex species have been found in a wide range of hosts. *Bacillus thuringiensis* colonises the guts of a wide range of invertebrates including insects belonging to the orders Coleoptera, Diptera and Lepidoptera (Jensen et al. 2003). *Bacillus cereus* has also been isolated in the gastrointestinal tracts of various arthropod species, such as soil-dwelling insects (Margulis et al. 1998) as well as mosquito larvae (Luxananil et al. 2001) and aphids (Jensen et al. 2003). *Bacillus anthracis* is considered to be the main mammalian pathogen within the *B. cereus* complex, with strains detected most often in the guts of herbivores (particularly different species of ungulates) and less often in certain omnivores and carnivores (Fasanella et al. 2010). Compared to *B. anthracis,* less is known about the ability of other *B. cereus* complex species to colonise mammalian hosts. Jadamus et al. (2001) found that probiotic strains of *B. cereus* (*Bacillus cereus var. toyoii*) spores are capable of germination in the upper intestinal tracts of chickens as well as piglets. Wilcks et al. (2008) dosed gnotobiotic rats with *B. thuringiensis* spores and found that spores were able to germinate and colonise the gastrointestinal tracts of these rodents. This led the authors to suggest that immune-compromised animals may be at risk of opportunistic gut colonisation by *B. cereus* complex species, although it should be noted that in both studies, *B. cereus* complex strains were artificially introduced into animal guts.

1.2.4.5. Ecology

Knowledge on the biological and ecological properties of *B. cereus* complex species is limited (Jensen et al. 2003). *Bacillus cereus* complex species have the ability to exploit a wide range of environments due to their capacity to utilise different organic and inorganic compounds whilst growing vegetatively (Zwick et al. 2012). These bacterial species can persist in unfavourable and stressful environmental conditions through spore formation (Von Stetten et al. 1999). Different studies confirm that members belonging to the *B. cereus* complex tend to prefer alkaline soils, high moisture content with high levels of organic matter (Dragon & Rennie 1995; Smith et al. 1999; Vilain et al. 2006; Blackburn et al. 2014). It has been shown both indirectly and experimentally that species within this bacterial complex may not have a direct affinity for alkaline soils, but rather require high levels of calcium to prevent the desiccation and promote the persistence of both spores and vegetative cells within soil environments (Dragon & Rennie 1995; Vilain et al. 2006).

It is disputed if *B. cereus* complex species are capable of a truly saprophytic lifestyle (Dragon & Rennie 1995; Vilain et al. 2006). Some argue that *B. cereus* complex species are unable to germinate in soil and either live pathogenic lifestyles, infecting mammals and invertebrates or live symbiotically within the rhizosphere by producing antibiotics which limit fungal growth and thereby promote plant growth (Vilain et al. 2006). Certain areas, such as the rhizosphere may provide microenvironments where *B. cereus* complex species can grow vegetatively (Brillard et al. 2015). Generally it is expected that with an increase in soil depth, there will be a decrease in overall bacterial abundance as carbon availability declines with increasing soil depth (Fierer et al. 2003). In contrast to this, Brillard et al. (2015) found that *B. cereus* complex species showed no difference in abundance at different soil depths, with *B. cereus* complex strains mostly being present as spores within the soil they tested. However, Vilain et al. (2006) were able to show that *B. cereus* strains are able to germinate and sporulate in liquid soil extracts, thus acting as true saprophytes. This raises an important question: Are pathogenic *B. cereus* complex species, such as *B. thuringiensis* as well as *B. anthracis* capable of the same saprophytic growth and therefore able to propagate and multiply outside their hosts?

Water movement through soils can result in the leaching of cations, such as calcium and as a result has an effect on the persistence of *B. cereus* complex species in specific soil environments (Dragon & Rennie 1995). Furthermore, a growing body of evidence suggests that

the atmosphere can act as a temporary environment in which certain microorganisms can exist, with studies finding evidence of microorganisms being present in both fog and rainfall (Amato et al. 2007). To date only one study conducted by Brillard et al. (2015) has looked at the effect of the water cycle on *B. cereus* complex ecology. This study found culturable *B. cereus* complex strains to be present in both rainwater as well as groundwater, suggesting that, if *B. cereus* complex species germinate in soil environments, contamination of both rainwater as well as groundwater can occur. This can lead to the propagation of *B. cereus* complex species in novel environments. Additionally, the study also found that the degree of contamination by *B. cereus* complex strains in groundwater varied between different rainfall events, leading the authors to suggest that groundwater contamination may be influenced by seasonal and climatic factors. Seasonality is also expected to influence *Bacillus* strain prevalence, with Sutherland and Murdoch (1994) finding a higher incidence of *B. cereus* complex species in pasteurised milk during summer months compared to winter months and suggesting that environmental factors, such as competitive exclusion by other *Bacillus* spp. in surrounding soil may be a contributing factor. Turnbull and Kramer (1985) found that faecal samples of people living in rural communities north-east and north-west of Johannesburg, South Africa showed a higher incidence of *B. cereus* strains during summer compared to winter. This geographical region is semi-arid and is a summer rainfall region.

Optimal growing temperatures of strains belonging to the seven phylogenetic clusters of the *B. cereus* complex vary (Table 1.3) (Guinebretière et al. 2010; Carlin et al. 2010). *Bacillus cereus* complex species are expected to show a higher diversity in temperate climates, high in temperature fluctuations, compared to climates with extreme temperatures, showing low fluctuations in mean temperature (Von Stetten et al. 1999). This increased diversity in temperate climates can be because the *B. cereus* complex comprises both mesophilic as well as psychrotolerant, *B. wheihenstephanensis,* strains (Guinebretière et al. 2010). Mesophilic *B. cereus* complex strains grow at temperatures above 7ºC and below 46ºC, while psychrotolerant strains tend to grow best at temperatures below 7ºC and up to 38ºC (Von Stetten et al. 1999). As a result, it has been suggested that in temperate climates with fluctuating average temperatures, selection will favour more than one thermal type, while in more extreme environments low in temperature fluctuations, only one thermal type will be favoured (Von Stetten et al. 1999).

1.2.4.6. Genetic studies on clinical samples

With the exception of some studies, such as that conducted by Sacchi et al. (2002), most genetic studies have tried to distinguish between *B. cereus* complex strains obtained from pure cultures. Sacchi et al. (2002) used a primer set targeting the 16S rRNA gene region to discriminate between different *B. cereus* complex species present in clinical samples. The 16S rRNA gene region has been shown to be too conserved across different *B. cereus* complex species to accurately differentiate between different species present in this group (Yamada et al. 1999). Because of the inability to directly and accurately distinguish between different *B. cereus* complex species present in environmental and clinical samples there is currently a dearth of knowledge regarding the natural reservoirs of *B. cereus* complex species, and the potential pathogenic capabilities of species belonging to this bacterial group. Because of the close association that bathyergids have with the rhizosphere where they obtain roots and tubers as food sources, accessible from underground burrows (Hubálek et al. 2005), they are an ideal candidate taxonomic group for investigating their suitability to act as sentinels for potentially pathogenic soil-associated bacteria. Their role as underground ecosystem engineers exposes them to a microclimate, which, is considered to be a hotspot for the evolution of opportunistic bacterial pathogens. As a result, it is likely that mole-rats harbour opportunistic pathogens which have evolved within the rhizosphere microclimate.

1.3. Blood-sucking arthropod-transmitted bacteria

1.3.1. Vector-borne diseases worldwide

In recent decades, more and more vector-borne diseases have emerged worldwide, raising public health concerns (Kilpatrick & Randolph 201). The persistent destruction of pristine environments and resultant reduction in biodiversity around the globe due to urbanisation, especially in developing regions such as southern Africa, is expected to increase the risk of humans contracting various vector-borne diseases (Ostfeld 2009). The reason for this is that in areas with low species richness, vectors only feed on a limited number of host species, whereas in areas high in species richness vectors come into contact with multiple hosts, which can vary in their capacity to act as carriers and reservoirs in the disease transmission of these pathogens (Bradley & Altizer 2007; Ostfeld 2009). Therefore, in natural areas, high in biodiversity, multiple hosts create a "dilution effect" on the transmission of vector-borne pathogens, whereas in urbanised areas, which

are often lower in species richness, the overall increased abundance of a limited number of host species, that are effective reservoir species of vector-borne diseases, can increase disease transmission (Bradley & Altizer 2007; Ostfeld 2009). As a result, the need for investigations into wildlife reservoirs which could be host to zoonotic pathogens and live in close association with human communities, amplifying the potential for disease transmission, is increasingly recognised (Kilpatrick & Randolph 2012).

Furthermore, modern globalisation and growing levels of trade and travel between different countries has led to the increased risk of introducing vector-borne pathogens into novel environments (Kilpatrick & Randolph 2012). These pathogens can rapidly become well-established and cause diseases in novel environments, as occurred when West Nile virus, introduced in New York City in 1999, subsequently spread across the United States of America within a few years of introduction (Ostfeld 2009). Moreover, with the ever-increasing expansion of human developments into natural environments, especially in resource-poor and developing countries, changes in landscape and poor socioeconomic conditions are expected to greatly increase the risk of endemic vector-borne pathogens emerging within these landscapes and opportunistically infecting human populations (Bradley & Altizer 2007; Kilpatrick & Randolph 2012). Changes in land use affect and alter interactions between potential natural reservoir hosts and their vectors as well as putting humans at risk of coming into contact with both reservoirs and their disease-carrying vectors (Kilpatrick & Randolph 2012). Furthermore, people living in poor socioeconomic environments often need to rely on alternative sources of protein, including various wildlife species, for subsistence or to supplement food sources. This dependence increases contact opportunities and exposure to reservoirs and their vectors (Kilpatrick & Randolph 2012). With the high HIV infection rates in southern Africa, and in South Africa in particular, susceptibility to possible zoonotic pathogens has increased in recent years (Comer 2001; Gopinath & Singh 2009). Moreover, as a result of various financial and technical constraints, the dynamics of various vector-borne diseases in many developing countries remain understudied and largely unknown (Kilpatrick & Randolph 2012). This highlights the need to develop cost-effective methodologies that will permit identification of potential zoonotic and vector-borne diseases, along with their hosts and vectors, to aid in early detection and prevention of disease in vulnerable human populations.

1.3.2. Haemotropic *Mycoplasma* species

Mycoplasma is a bacterial genus closely related to gram-positive bacteria such as streptococci, bacilli and lactobacilli (Atkinson et al. 2008). By far the smallest within this group, both in diameter and genome size, more than 200 species of *Mycoplasma* are known to associate with plants, humans and animals, most often co-existing within hosts without causing infection (Messick 2004; Atkinson et al. 2008). However, more and more cases have been reported where both previously healthy patients as well as immune-compromised patients, show signs of latent infections (Messick 2004). *Mycoplasma* species can cause a range of diseases, such as respiratory infections, infections of the central nervous system as well as infections in the genito-urinary tract, which in some cases can prove difficult to treat (Dallo & Baseman 2000).

Haemotropic *Mycoplasma* species (haemoplasmas) are gram-positive, obligate erythrocyte pathogens that have not been cultured in cell-free media to date (Messick 2004). Haemoplasmas differ significantly from other *Mycoplasma* species when looking at the 16S rRNA gene region (Santos et al. 2009). Haemotropic *Mycoplasma* species lack cell walls and have circular double-stranded DNA which only encodes genes that are necessary for life, virulence and pathogenicity (Bosnic et al. 2010; Santos et al. 2009). Recently the whole genomes of both *M. suis* and *M. haemofelis* were characterised, revealing that these haemoplasma strains not only have smaller genomes than their *Mycoplasma* relatives, but that both haemoplasma species have large numbers of variable number tandem repeats (VNTRs) in their genomes, which may enable these bacterial pathogens to adapt quickly to changing environments (Santos et al. 2009). The *M. haemofelis* genome was shown to encode a number of unique proteins, suggesting that these proteins may allow for the adaption to a blood environment (Santos et al. 2009).

1.3.2.1. Taxonomy

Similar to *B. cereus* complex species, haemoplasmas have had an uncertain taxonomic history. Initially, haemotropic *Mycoplasma* species were classified into two different genera, *Haemobartonella* and *Eperythrozoon* and were thought to belong to the family Anaplasmataceae within the order Rickettsiales (Messick 2004; Sykes 2010). This classification was based on biological evidence, in that species belonging to these genera were very small in size, were obligate parasites and had a close association with red blood cells (Messick 2004; Neimark et al. 2004). However, others argued that these species were more closely related to members of the class

Mollicutes (which consists of eight genera: *Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma, Anaeroplasma, Asteroleplasma, Mesoplasma*, and *Entomoplasma*) (Messick 2004). This suggestion was based on characters such as the lack of a cell wall and flagella, inability to parasitise cells intercellularly, small organismal and genomic size as well as resistance to penicillin, but susceptibility to tetracycline (Messick 2004; Willi et al 2007a). Phylogenetic analyses of the 16S rRNA gene region confirmed that species initially classified within the *Haemobartonella* and *Eperythrozoon* genera formed a part of the *Mycoplasma* genus and are now known as haemotropic *Mycoplasma* species (Messick 2004; Neimark et al. 2004; Sykes 2010; Fard et al. 2014). Both 16S rRNA data and their unique tropism for erythrocytes place haemoplasmas in their own phylogenetic clade within the *Mycoplasma* genus and two phylogenetic clusters, the haemofelis cluster and the suis cluster of haemoplasma strains are currently recognised (Santos et al. 2009). These two phylogenetic clusters seem to share a common ancestor and are most closely related to the pneumonia group of *Mycoplasma* strains (Santos et al. 2009).

1.3.2.2. Diversity, hosts, pathogenicity and transmission

The first haemotropic strain (then classified as *Eperythrozoon felis*) was detected in 1942 in an anaemic cat from South Africa (Sykes 2010). Today, haemoplasma strains have been found worldwide and as no haemoplasma strains have been cultivated *in vitro*, and microscopy has proven too insensitive to confirm haemoplasma presence, the most accurate way to detect these pathogenic bacterial strains is through bacterial genome amplification (Grazziotin et al. 2011; Barker & Tasker 2013). To date various haemoplasma species have been classified based on 16S rRNA data and species belonging to this bacterial group seem to have co-evolved with a wide range of mammalian hosts including humans (Sykes 2010), domestic as well as wild felids (Willi et al. 2007a, 2007b), various species of rodents (Messick 2004; Vieira et al. 2009), camels (Nazifi et al. 2009), dogs (Kenny & Shaw 2004), cattle (Tagawa et al. 2008), pigs (Yuan et al. 2009), sheep and goats (Neimark et al. 2004). Clinical symptoms can vary from asymptomatic to severe haemolytic anaemia, with the latter being due to direct tropism of red blood cells or through immune response breakdown of infected erythrocytes (Novacco 2012). This haemolytic anaemia can be fatal depending on the specific infecting species as well as the host's immune-competence (Willi et al. 2007b). Although no clear distinction between the acute and chronic phase exists, the acute phase is characterised by anorexia, lethargy, dehydration, weight loss and pyrexia (Novacco

2012). It has been postulated that certain hosts, such as cats and rodents may become clinically healthy after initial infection, acting as carriers of haemoplasma strains which could then potentially be passed on to other individuals by blood-sucking vectors. Host immune-competence seems to play an important role in haemoplasma prevalence and some felid-associated strains have been found to co-infect hosts along with retroviruses, such as feline immunodeficiency virus (FIV) (Santos et al. 2009; Barker & Tasker 2013). Furthermore, hosts, especially felids, are often co-infected with more than one haemoplasma strain, complicating understanding of the specific pathogenicity of each strain (Barker & Tasker 2013).

Haemoplasmas are considered to be transmitted from one host to another through different blood-sucking arthropod vectors (Willi et al. 2006; Goncalves et al. 2015). Species of lice (*Polyplax serrata* and *P. spirulosa*) and fleas (*Synosternus cleopatrae*) have been identified as vectors for haemoplasma strains, but the possibility that other blood-sucking arthropods, such as ticks and mites, may act as vectors for haemoplasma strains remains to be elucidated (Goncalves et al. 2015). Warmer climates should have a higher incidence of haemoplasma infection as potential arthropod vectors tend to have a higher abundance in warmer climates compared to colder climates (Willi et al. 2006). Moreover, it has been shown indirectly that for areas higher in rainfall there is a higher haemoplasma incidence compared to more arid areas. This is because rainfall can have an effect on vector developmental stages and therefore vector abundance (Goncalves et al. 2015). These authors also suggest that seasonality, as well as relative humidity, may have similar impacts on arthropod abundance in specific areas which could again influence haemoplasma incidence. It is also posited that changes in host behaviour, such as behavioural adjustments during mating season or fluctuations in host density, will impact the level of haemoplasma prevalence as reproductive activities and increased interactions between conspecifics in high density populations will result in higher levels of ectoparasite exchanges between individuals (Archer et al. 2014; Lutermann et al. 2015). Although other transmission routes are poorly understood it has also been suggested that haemoplasma strains can be transmitted vertically and, as haemoplasma DNA has been found in saliva and faeces of different hosts, animal-to-animal contact such as fighting is expected to result in effective transmission (dos Santos et al. 2008; Grazziotin et al. 2011; Maggi et al. 2013a).

1.3.2.3. Haemoplasma in rodents

Knowledge of haemoplasma strain diversity, prevalence, distribution and bacterial-host-vector interactions are limited, and with the continual identification of novel strains, many of which hold zoonotic potential, concern about this lack of knowledge has been raised (Goncalves et al. 2015). Members belonging to the order Rodentia, the most diverse and widespread order of all mammals have been identified as possible carriers of haemoplasmas (Goncalves et al. 2015). However, the zoonotic potential of these rodent-infecting haemoplasmas has not been established, and the prevalence of haemoplasmas in wild rodent populations is severely under-documented (Sashida et al. 2013). Two recognised *Mycoplasma* species, *M. haemomuris* and *M. coccoides* are known to infect laboratory and wild rodent populations and are likely transmitted from one host to another by different species of lice (Biondo et al. 2009; Vieira et al. 2009). Depending on the host species inhabited, *M. haemomuris* is separated into two subspecies, *viz. M. haemomuris* subsp. *musculi* and *M. haemomuris* subsp. *ratti* (Goncalves et al. 2015). Recently, novel rodent haemoplasma strains have been identified in capybaras (*Hydrochaeris hydrochaeris*) in Brazil (Vieira et al. 2009), free-living brown sewer rats (*Rattus norvegicus*) in Morioka, Japan (Sashida et al. 2013), free-living house mice (*Mus musculus*), brown rats (*Rattus norvegicus*) and a harvest mouse (*Micromys minutus*) in Hungary (Hornok et al. 2015), as well as in various wild-caught rodent species across Brazil (Goncalves et al. 2015). This suggests that rodent haemoplasma strains may be much more genetically diverse and widespread than previously thought. Although little is known about haemoplasma prevalence in wildlife, free-living populations have been shown to have higher haemoplasma infection rates compared to their captive-held con-specifics (Vilain et al. 2006). This further emphasises the need to better understand haemoplasma prevalence and diversity in free-ranging host species.

1.3.2.4. Haemoplasma strains in South Africa

Although first discovered in South Africa, studies on haemoplasma prevalence in this country are limited. There have been further reports of haemoplasma strains in domesticated cats from this region (Tasker et al. 2003; Willi et al. 2006), with one study reporting an overall haemoplasma prevalence of 26% in domestic cat samples tested (Willi et al. 2006). Two reports of South African patients being infected with haemoplasma strains also exist (Tasker et al. 2010;

Maggi et al. 2013c). In the first report, Tasker et al. (2010) screened the blood samples of 100 HIV-positive patients from South Africa for haemoplasma prevalence using a qPCR assay and found one of the samples positive for a haemoplasma strain. Maggi et al. (2013c) used PCR assays to detect "*Canditatus* Mycoplasma haematoparvum" from blood samples of a veterinary surgeon who reported contact with a wide range of South African mammals and their ectoparasites. This patient was also co-infected with *Bartonella henselae* and *Anaplasma platys* and showed clinical symptoms including severe migraines, fainting, seizures and neurocognitive abnormalities. This was the first report of "*Canditatus* Mycoplasma haematoparvum" in human blood, a haemoplasma strain normally associated with domesticated dogs (Sykes et al. 2005). However, data on haemoplasma strain diversity and distribution are limited and data on potential reservoirs of these strains, such as indigenous rodent communities, are generally depauperate.

1.3.2.5. Influence of human-transformation

Moreover, the level of human transformation of areas occupied by rodent species may have an effect on the level of haemoplasma prevalence. Goncalves et al. (2015) postulated that in areas less disturbed by anthropogenic modification indigenous rodent species may show lower levels of haemoplasma infections compared to indigenous rodent species present in human-transformed environments. The reason for this is that in human transformed areas synanthropic rodent species, such as *Rattus rattus* and *R. norvegicus,* occur in these areas due to historic anthropogenic introduction. These authors hypothesised that synanthropic rodent species, introduced during European colonisation may have already been infected with haemoplasma strains. As a result, the possibility exists that in human transformed areas where synanthropic rodents co-occur with indigenous rodent species, exchange of haemoplasma pathogens through different arthropod vectors shared between these exotic and indigenous rodent populations is possible. This could then result in higher haemoplasma prevalence in indigenous rodent populations occurring in transformed areas compared to their conspecifics inhabiting natural environments.

1.3.2.6. Inter-species transmission

Phylogenetic analyses have shown that "*Candidatus* Mycoplasma turicensis", a species associated with causing haemolytic anaemia in both domesticated and wild cats, and previously detected in domesticated cats sampled in Johannesburg South Africa (Willi et al. 2006), is more

closely related to *M. haemomuris* and *M. coccoides* than to other haemoplasmas infecting felids (Willi et al. 2007a). This has led to the hypothesis that, although haemoplasmas have traditionally been thought of as being host-specific, some strains, typically associated with rodents, may be capable of interspecies transmission to other mammalian hosts, although the mechanism of this interspecies transmission remains to be elucidated (Willi et al. 2007a; Maggi et al. 2013a).

Nevertheless, this does highlight the potential for these rodent-borne haemoplasma strains to move between different host species. Furthermore, a growing body of literature suggests that haemoplasmas may represent a group of newly emergent zoonotic pathogens (Hu et al. 2004; Neimark et al. 2004; Sykes 2010; Maggi et al. 2013a; Barker & Tasker 2013; Fard et al. 2014). It has been shown genetically that the haemoplasma strain identified in humans, "*Candidatus* Mycoplasma haemohominis", is most closely related to *M. haemomuris* (Hornok et al. 2015). Reports also exist confirming co-infection of haemoplasma-positive patients with other haemotropic bacteria, such as *Bartonella henselae* (Dos Santos et al. 2008; Sykes et al. 2005). Haemoplasma strains seem to be opportunistic human pathogens, with immune-compromised humans who have close associations with wild and domestic animals considered to be at higher risk of becoming infected with different haemoplasma strains (Barker & Tasker 2013; Maggi et al. 2013a). Taking this into consideration, and given that haemoplasma presence was previously confirmed in African mole-rat species by broad-range 16S rRNA PCR and sequencing (Table 1.2; Retief 2014), a more detailed investigation of haemoplasma in bathyergids is warranted.

1.3.2.7. Haemoplasma detection

Genomic amplification by means of the polymerase chain reaction (PCR) has been shown to be a fast and accurate diagnostic method that is particularly valuable for confirming the presence of bacterial species that are difficult to culture, in clinical and environmental samples (Gopinath & Singh 2009; McAuliffe et al. 2013). Moreover, traditional taxonomic classification of bacterial species relies on metabolic, morphological and physiological data, which requires the isolation and cultivation of the bacterial species under question (Kent & Triplett 2002). However, as less than 2% of bacterial species present in environmental samples are likely to be successfully cultivated when using traditional, culture-based methods, alternative approaches for accurate assessment of bacterial diversity are required (Kent & Triplett 20002; Wade 2002). To date attempts at cultivating haemoplasma strains have been unsuccessful (Messick 2004; Willi et al.

2007b). This inability to cultivate these bacterial strains has resulted in a limited understanding of both the biology and pathogenesis of these infectious agents and has also impeded the development of accurate haemoplasma strain detection methods. Historically, detection strategies have involved microscopic identification, an approach which lacks both sensitivity and specificity (Santos et al. 2009). Methods which prove useful for assessing genetic diversity in other bacterial genera, such as DNA:DNA hybridisation are not feasible for haemoplasma strains as these methods require large amounts of DNA, for which culturing is necessary (Tasker et al. 2003). As a result, PCR techniques are currently seen as the gold standard for accurately identifying haemoplasma strains present in different hosts (Conrado et al. 2015). Therefore, using PCR-based methods to better understand the prevalence and diversity of these pathogens in bathyergids is the best approach in attempting to bridge the knowledge gap that currently exists for wildlife reservoirs of this bacterial genus.

1.4. Aims

The purpose of the current study was to gain a better understanding of bacterial species present within bathyergid hosts living in the south-western part of South Africa. Molecular techniques were applied to detect and characterise the specific *B. cereus* complex species present in different species of bathyergids, allowing for the identification of *B. cereus* complex lineages in environmental or clinical samples. The molecular approach employed enabled assessment of the sentinel potential of bathyergid species for anthropogenic soiling, whilst at the same time optimising available *B. cereus* complex assays. This together with the development of two novel assays ensured rapid and accurate detection of strains in environmental and clinical samples. Molecular techniques were also used to test the accuracy of three different PCR assays for detecting haemoplasma strains present in bathyergids. The aim here was to assess both haemoplasma prevalence and strain diversity in wild-caught and captive-held bathyergid species, whilst simultaneously optimising 16S rRNA PCR assays for improved detection of haemoplasma strains in environmental and clinical samples.

1.5. Research questions

1.5.1 Are bathyergids sentinels of potentially pathogenic bacterial strains belonging to the *B. cereus* complex?

1.5.1.1 Is there a difference in prevalence of *B. cereus* complex strains between different bathyergid species and between bathyergids sampled in an urban setting compared to bathyergids sampled in undisturbed / natural settings?

1.5.1.2 Can *B. cereus* complex strains be detected in clinical or environmental samples and characterised to species level using available conventional PCR- sequencing techniques?

1.5.2 What is the diversity and prevalence of haemoplasma strains in bathyergids?

1.5.2.1 How does haemoplasma strain diversity and prevalence vary between different host species and area of sampling of bathyergid hosts?

1.5.2.2 Can conventional PCR assays accurately detect and characterise haemoplasma strains in bathyergid samples?

1.6 Hypotheses

The two main research questions posed above were addressed individually in two separate research chapters and given the above questions the following two hypotheses were tested in this study:

Hypothesis 1.6.1

Hypothesis 1.6.1.1

H_0: There is no significant difference in *B. cereus* complex strain prevalence between the four bathyergid species and between bathyergids sampled in an urban setting and bathyergids sampled in natural settings.

H_1: There is a pronounced difference in *B. cereus* complex strain prevalence between the four bathyergid species and between bathyergids sampled in an urban setting and bathyergids sampled in natural settings.

Hypothesis 1.6.1.2

H_0: Conventional PCR molecular techniques are unable to accurately detect and characterise *B. cereus* complex species present in clinical or environmental samples.

H_1: Conventional PCR molecular techniques are useful for detecting and characterising different *B. cereus* complex species present in clinical or environmental samples.

Hypothesis 1.6.2

Hypothesis 1.6.2.1

H_0: There is no significant difference in haemoplasma strain diversity and prevalence between the four bathyergid species sampled (*B. suillus, G. capensis, C. h. hottentotus* or *F. damarensis*), nor between different sampling localities.

H_1: There is a significant difference in haemoplasma strain diversity and prevalence between the four bathyergid species sampled (*B. suillus, G. capensis, C. h. hottentotus* or *F. damarensis*) and between different sampling localities.

Hypothesis 1.6.2.2

H_0: Conventional PCR molecular techniques are unable to accurately assess the diversity and prevalence of haemoplasma strains present in bathyergid samples in South Africa.

H_1: Conventional PCR molecular techniques are valuable for assessing the diversity and prevalence of haemoplasma strains present in bathyergid samples in South Africa.

1.7 **Relevance of study**

Consideration needs to be given to the problem of human settlements that are continuously encroaching into natural environments thereby increasing the risk of people coming into contact with animals and environments harbouring pathogens they would not have readily encountered before, resulting in emergence / re-emergence of disease (Comer 2001). This, together with the high proportion of immune-compromised individuals present in poorer communities of South Africa (Kenyon et al. 2013), highlights the need to better understand the diversity and distribution of potential pathogens present in South Africa. *Bathyergus suillus* and its close relatives are potential natural reservoirs of various bacterial pathogens. This is of particular concern as many

people living in rural communities catch and eat this rodent species, a practice that may facilitate the spread of potentially zoonotic pathogens.

Bacillus cereus complex species have been found worldwide in different environments (Jensen et al. 2003; Tourasse et al. 2006). Most of the species belonging to this bacterial complex have the potential to act as human pathogens, with pathogenicity varying from species to species (Tourasse et al. 2006). Moreover, studies reporting the detection and characterisation of *B. cereus* complex species from environmental samples are limited (Jensen et al. 2003).

It is increasingly recognised that anthropogenic activities, such as bioremediation or pollution through waste water runoff, not only alter the soil microbiota community structure, but also introduce opportunistic bacterial pathogens into human transformed systems. Considering these factors, along with the potential pathogenicity of *B. cereus* complex strains (Mandic-Mulec et al. 2015), this study aimed to gain a better understanding of the dynamics of *B. cereus* complex species in South Africa. Moreover, by looking at *B. cereus* complex strain prevalence in both urban and naturally sampled bathyergid species, this study assessed the potential of urban wildlife to act as sentinels for anthropogenic soiling. The molecular techniques used in this study represent the first attempt to detect and characterise *B. cereus* complex species present in free-ranging mammals, across a range of human-transformed environments.

Considering that more than half of emergent and re-emergent bacterial pathogens are expected to arise from developing regions such as southern Africa, investigating relatively neglected vector-borne bacterial pathogens with possible zoonotic potential is essential (Gratz 1999; Blancou et al. 2005). Bearing in mind that haemotropic *Mycoplasma* species have potential as zoonotic pathogens (Sykes 2010) and are found in a range of rodent species (Messick et al. 2004; Vieira et al. 2009), investigating the potential of bathyergid species to be hosts to these pathogens is warranted. As limited sequence, distribution and diversity data are available (Willi et al. 2007a) and as these pathogens can result in severe disease and even death (Fard et al. 2014), it is important to develop an assay capable of accurately detecting and discriminating between different haemoplasma strains. The PCR assays assessed in this study aid in detecting bathyergids infected with these pathogens along with gaining a better appreciation of haemoplasma strain diversity within bathyergids occurring in the south-western part of South Africa.

1.8 **Chapter outline**

The structure of this dissertation follows the structure of the two main research questions posed. In this first chapter, an overall review of the literature available on South African bathyergids, the *B. cereus* complex and haemotropic *Mycoplasma* species is provided. Specifically, this chapter summarises what is known of species belonging to the two different bacterial genera and why investigating the prevalence and diversity of these bacterial genera within bathyergid lungs is important, whilst highlighting the knowledge gaps that limit our understanding of the dynamics of these bacterial species.

In the second chapter, the ability to detect and characterise *B. cereus* complex species in environmental samples is assessed using published conventional PCR molecular methods. *Bacillus cereus* complex prevalence and diversity is evaluated in four bathyergid species, namely *B. suillus, G. capensis, C. h. hottentotus* and *F. damarensis*. These bathyergid species were sampled in various locations in the south-western part of South Africa. Standard phylogenetic and statistical techniques were employed to determine the intra- and inter-specific diversity of *B. cereus* complex species in four bathyergid species and how the prevalence of these *B. cereus* complex species differ between different species, sexes and area of sampling of bathyergid hosts. The data generated in this chapter assist in evaluating the potential of urban bathyergids to be sentinels of anthropogenic soiling.

In the third chapter, the sensitivity and specificity of haemotropic *Mycoplasma* strain detection is assessed using three PCR assays. Results obtained with a published conventional PCR technique, are contrasted with the results obtained with two novel assays, each of which incorporate at least one primer, designed specifically for this study. These comparisons allow for assessment of the comparative utility of three 16S rRNA gene-targeting assays, and ultimately enable rapid and accurate detection and discrimination of haemoplasma species present within various tissues of the four bathyergid species evaluated in this study, namely *B. suillus, G. capensis, C. h. hottentotus* and *F. damarensis*. In combination with nucleotide sequencing, the PCR assay developed in this chapter aids in improving our knowledge of haemoplasma strain distribution and diversity in subterranean wildlife populations in southern Africa.

The final chapter serves as a general conclusion, and recommendations are made for future research based on the findings of this study.

1.9 **Methodology**

The prevalence of two potentially pathogenic bacterial groups, *viz.* soil-borne *B. cereus* complex strains and vector-borne haemotropic *Mycoplasma* strains, in four mole-rat species all belonging to the family Bathyergidae (Table 1.4; Figure 1.2), was evaluated in this study.

Table 1.4: Sampling information for four bathyergid species evaluated in the current study.

Bathyergid species	Sample site	Co-ordinates	Habitat type	Ethics clearance
Bathyergus suillus	Cape Town airport, Western Cape	33°58'10''S 18°35'50''E	Urban	AUCC 040702-015
Cryptomys hottentotus	Darling, Western Cape	33°25'S 18°25'E	Semi-natural	EC005-11
hottentotus	Kamieskroon, Northern Cape	30°13'S 17°57'E	Rural	EC005-11
Georychus capensis	Darling, Western Cape	33°25'S 18°25'E	Semi-natural	EC118-13
Fukomys damarensis	Hotazel, Northern Cape	27°17'S 22°58'E	Natural	AO27/06

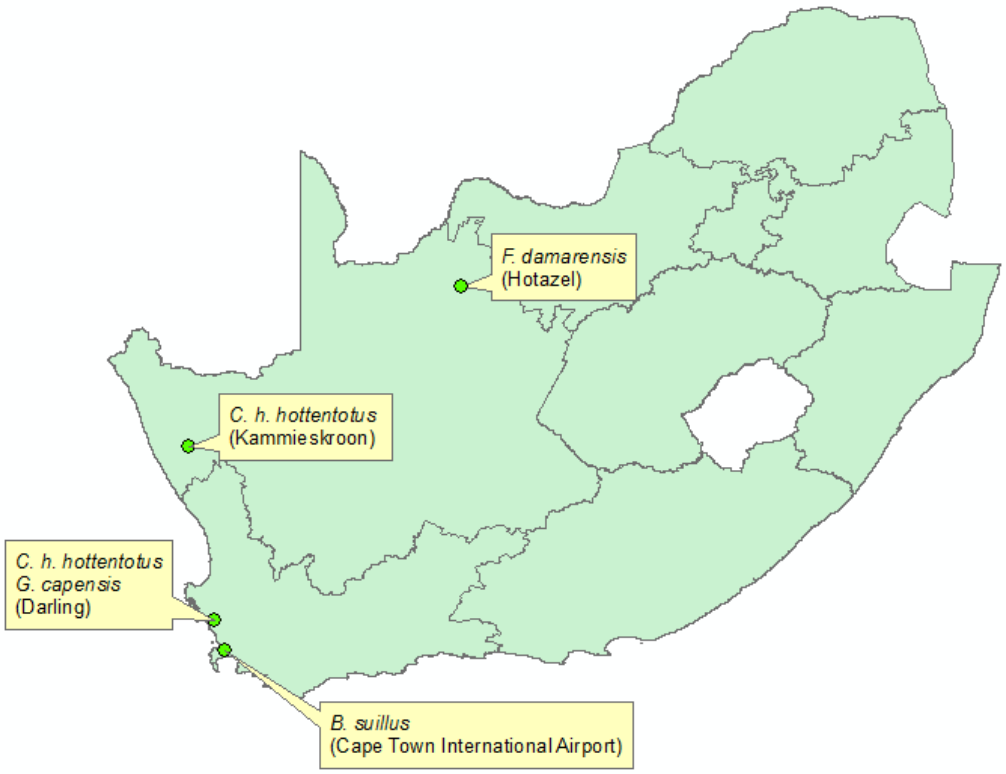

Figure 1.2: Map indicating sampling localities of four bathyergid species screened for bacterial prevalence in the current study.

For the first data chapter, lung samples from four mole-rat species, belonging to the family Bathyergidae were evaluated. Three species were trapped using Hickman traps, while *Bathyergus suillus* was captured using modified snap traps. All animals were euthanised by halothane overdose as prescribed by the ethics committee of the University of Pretoria. Genomic DNA extractions performed on dissected lung tissue samples stored in absolute ethanol are all bi-products from prior studies. In the first data chapter, the extracted DNA samples from 234 animals were screened for the presence of *B. cereus* complex species using seven published primer sets (denoted A-G) targeting the 16S rRNA (A), *yea*C (B), *gro*EL (C) and *gyr*B (D-G) gene regions. Additional screening with two newly designed primer sets (H1 and H2), both targeting the *gro*EL gene region, permitted determination of the levels of co-infection in samples positive for *B. cereus* complex strains. All samples with amplicon sizes of the expected size were purified and cycle sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Nucleotide BLAST (Altschul et al. 1990) searches were performed to identify closely related sequences in the GenBank database (www.ncbi.nlm.nih.gov/blast). Nucleotide sequences generated in the course of this study were edited and aligned with valid reference sequences obtained from the GenBank database (www.ncbi.nm.nih.gov/genbank) using ClustalW (in MEGA 6). Neighbor joining (NJ) analyses were performed using MEGA 6 (Saitou & Nei 1987; Tamura et al. 2013). Appropriate best-fit models were identified in Mega 6 under the Bayesian Information Criterion (BIC). The best-fit model identified in this manner for each of the datasets was used for minimum evolution (Nei & Kumar 2000) analyses in Mega 6 and Maximum likelihood analysis (performed in phyML) (Guindon et al. 2010), and guided the selection of priors for Bayesian inference (performed in MrBayes) (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Differences in *Bacillus* prevalence between host species, sexes and level of urbanisation where bathyergid species were sampled were analysed using Pearson Chi-square tests in IBM SPSS.

For the second data chapter, lung, liver and spleen samples from members belonging to the family Bathyergidae were evaluated for *Mycoplasma* genome presence. Wild-caught *Fukomys damarensis, C. h. hottentotus* and *Georychus capensis* individuals were trapped using Hickman traps while wild-caught *Bathyergus suillus* individuals were captured using modified snap traps. Both wild-caught and captive-held animals were euthanised by halothane overdose as prescribed by the ethics committee of the University of Pretoria. DNA extracted from five flea samples and

two lice samples, all belonging to the family Hystrichopsyllidae and found on *B. suillus* were made available for this study and were bi-products from prior studies. The relative sensitivity and specificity of three PCR assays, all targeting the 16S rRNA gene region, was calculated to identify the assay that is best-suited to accurate detection and characterisation of haemotropic *Mycoplasma* strains present in bathyergid lungs. All results were verified through purification and nucleotide sequencing of all amplicons of the expected size using the same methods described for the first data chapter. Subsequently, phylogenies and statistical analyses were also inferred in the same manner as described for the first data chapter.

Chapter 2

Subterranean mammals: reservoirs of infection or sentinels of anthropogenic soiling?

Abstract

Global reports of emergent pathogens in humans have intensified efforts to identify wildlife reservoirs. Subterranean mammals, such as bathyergid mole-rats, are largely overlooked, despite their high-level exposure to soil-dwelling microbes. In assessing bathyergid reservoir potential, we targeted the *Bacillus cereus* complex, a ubiquitous bacterial assemblage, comprising species with pathogenic and zoonotic potential. Microbe-bathyergid interactions were evaluated by sampling 234 bathyergids across a range of human-transformed landscapes in the Western Cape Province of South Africa. Four bathyergid species were screened for *Bacillus* genome presence using seven published and two novel PCR assays targeting four gene regions. Nucleotide sequencing confirmed the presence of two discrete *B. cereus* complex lineages. Overall *Bacillus* presence was 17.95%, ranging from 0% for *Georychus capensis* to 45.35% for *Bathyergus suillus*. *Bacillus* occurrence differed significantly between host species ($\chi 2 = 69.643$; $df = 3$; $p < 0.05$) and between sampling locality type ($\chi 2 = 70.245$; $df = 3$; $p < 0.05$), being significantly higher in bathyergids sampled from the peri-urban locality. The results highlight the sentinel potential of soil-dwelling mammals occurring in close proximity to informal human settlements and stress the importance of monitoring anthropogenically-introduced, opportunistic pathogens and the threats they pose to vulnerable communities, particularly in the developing world.

Key Words: Anthropogenic activities, urban wildlife, developing countries, Bathyergids, *Bacillus cereus* complex

Some of the results presented in this chapter have been included in a manuscript submitted to *EcoHealth* for possible publication.

2.1. Introduction

Members belonging to the family Bathyergidae (African mole-rats) are subterranean rodents, with high levels of exposure to a broad range of soil-dwelling microbes (De Graaff 1981). Bathyergids are underground ecosystem engineers, building extensive burrowing systems and living in close association with the rhizosphere, the microenvironment connecting soil and plant roots, where they obtain roots and tubers as food sources (Berg et al. 2005; Hubálek et al. 2005; Thomas et al. 2009). In pristine environments, with limited human impact, the microbe-bathyergid associations in the subterranean environment are ancient, whereas, in human transformed areas, newly-established microbe-bathyergid interactions will likely reflect as high-level infections in these naïve vertebrates. Opportunistic pathogens exist naturally in soil or result from anthropogenic introduction, either intentionally, through practices such as bio-remediation, or accidentally, through the pollution of soil systems (Baumgardner 2012; Duran-Alvarez & Jimenez-Cisneros 2014). Of growing concern is that wastewater, released into the environment, supplies a constant influx of both antibiotic-resistant pathogens and antibiotic-resistant genes to soil systems, which could potentially be transferred to other soil bacteria through horizontal gene transfer (HGT) in human transformed areas (Duran-Alvarez & Jimenez-Cisneros 2014).

The recent surge in opportunistic pathogens affecting immune-compromised patients, made more susceptible due to diseases such as HIV, cystic fibrosis or healthcare-associated infections, has raised public health concerns worldwide (Berg et al. 2005; Aujoulat et al. 2012). Microorganisms are significantly influenced by their environment, with many emergent pathogens belonging to soil-associated genera, forming bivalent interactions, growing both in the rhizosphere and opportunistically infecting animal and human hosts (Wilson and Slayers 2003; Berg et al. 2005; Aujoulat et al. 2012). Bacterial genera falling into this category include, among others, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus* and *Stenotrophonomans* (Berg et al. 2005; Aujolat et al. 2012). The nutrient-rich rhizosphere, considered a "hotspot" for the evolution of opportunistic bacterial pathogens, creates a microclimate where competition, infection and predation between bacteria, fungi, archaea, viruses and protozoa is intense (Berg et al. 2005; Aujoulat et al. 2012; Baumgardner 2012). Selection processes acting in this environment can predispose bacterial species to become pathogenic should they encounter humans or other animals (Berg et al. 2005; Aujoulat et al. 2012). HGT is facilitated in the rhizosphere and, in the presence of pathogenicity islands, may further

select for opportunistic pathogens within this environment (Berg et al. 2005; Aujoulat et al. 2012; Duran-Alvarez and Jimenez-Cisneros 2014).

Up to 30% of people living in and around Cape Town in the Western Cape Province of South Africa are HIV-positive (Kenyon et al. 2013), and the majority live in resource-poor settings often relying on less conventional sources of protein, including capture and consumption of Cape dune mole-rats, *Bathyergus suillus* (De Graaff, 1981). It is increasingly recognised that immune-compromised individuals are highly susceptible to pathogenic and innocuous microorganisms (Comer 2001; Berg et al. 2005; Kenyon et al. 2013). As underground engineers that live in a range of human-transformed landscapes, bathyergids potentially play a largely overlooked role as reservoirs of infection or as sentinels for the degree of microbial soiling of soil environments, through the activities of man.

A reservoir of infection is defined as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined population" (Haydon et al. 2002). In the same vein, sentinel surveillance acknowledges that populations of animals can be epidemiologically connected and uses this knowledge for cost-effective disease detection (Halliday et al. 2007). Crucially sentinel surveillance acts as an early-warning system and, in contrast to merely identifying a potential reservoir species, can also provide important information of potential environmental health hazards (Halliday et al. 2007).

Concern has been raised about the potential pathogenic bacterial genera present within natural water systems in the Western Cape Province of South Africa (Jackson et al. 2009; Paulse et al. 2012). In 2012, Paulse and colleagues investigated the bacterial community structure present in the Berg and Plankenburg rivers through 16S rRNA sequencing of bacterial isolates which included, among others, potentially pathogenic bacterial strains belonging to the *Bacillus cereus* complex from the Berg River. This water system serves as a water resource for various industrial, agricultural, domestic and municipal activities and, as one of the major avenues of soil pollution is contamination by wastewater, the surrounding soil systems likely also harbour these opportunistic pathogens (Jackson et al. 2009; Paulse et al. 2012; Duran-Alvarez & Jimenez-Cisneros 2014).

The *Bacillus cereus* complex is a ubiquitous, highly homogeneous assemblage of gram-positive, spore-forming, soil-borne bacterial species of contentious taxonomy (Jensen et al. 2003;

Thorsen et al. 2006; Tourasse et al. 2006; Didelot et al. 2009). To date, nine species of medical and economic concern have been recognised, *viz. B. cereus, B. anthracis, B. thuringiensis, B. bombysepticus, B. cytotoxicus, B. toyonensis, B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* (Jensen et al. 2003; Thorsen et al. 2006; Tourasse et al. 2006; Guinebretière et al. 2013; Jiménez et al. 2013; Cheng et al. 2014). Of these, three are of public health concern: (i) *Bacillus cereus,* causing emetic and diuretic food poisoning in humans, is a common soil inhabitant and a well-known food-spoiling organism (Didelot et al. 2009), (ii) *Bacillus anthracis* infects a wide range of mammalian hosts, including humans, causing anthrax, an often fatal haemorrhagic fever and potential bioterrorism agent (Fasanella et al. 2010) and (iii) *Bacillus cytotoxicus,* a thermotolerant species that produces an enterotoxin, cytokine K, which results in severe diarrhoeic food-poisoning (Tourasse et al. 2011; Guinebretière et al. 2013). The remaining species are either considered non-pathogenic (Ahmod et al. 2011) or have insect biological control applications (Hendriksen et al. 2006; Cheng et al. 2014).

Owing to the high levels of genetic similarity displayed within the *B. cereus* complex, a range of targets, including plasmid encoded gene regions, such as pXO1 and pXO2 and chromosomal markers, such as 16S rRNA, 23S rRNA, the 16S–23S rDNA intergenic spacer region, *rpo*B, *sasp*B and *plc*R transcriptional activator gene regions, have been evaluated (Bavykin et al. 2004; Tourasse et al. 2006; Park et al. 2007; Kim et al. 2008; Didelot et al. 2009; Rao et al. 2010; Irenge et al. 2010; Ahmod et al. 2011; Dzieciol et al. 2013). However, all failed to definitively distinguish between species belonging to this monophyletic cluster. Phylogenetic analyses are further complicated by the possibility that plasmid-encoded virulence factors can be transferred from one *B. cereus* complex species to another through HGT (Ceuppens et al. 2013).

A number of PCR assays are available for detection and characterisation of members of the *B. cereus* complex. Park et al. (2007) designed a multiplex PCR approach targeting the heat shock operon (*gro*EL) and the gyrase B (*gyr*B) gene regions to distinguish between the species in this complex. Ahmod et al. (2011) developed a primer set targeting the large conserved indel, *yea*C, to accurately distinguish *B. anthracis* strains from other *B. cereus* group species. However, both studies made use of pure cultures of type strains (Park et al. 2007; Ahmod et al. 2011) or those prepared from food samples (Park et al. 2007) to evaluate their assays, and neither approach has been evaluated using clinical or environmental samples. Considering that *B. cereus* complex species are soil inhabitants, found in a wide range of environments, optimisation of available

assays for enhanced assessment of *Bacillus* in environmental and clinical samples is important. The results will contribute to the generation of much-needed data on *B. cereus* complex species in their natural environment (Jensen et al. 2003) from a region of the world that is understudied, yet where knowledge of the occurrence and diversity of this bacterial genus is important.

2.2 Methods

2.2.1. Study sites, samples and bacterial 16S rRNA characterisation

Lung samples from four mole-rat species, belonging to the family Bathyergidae, collected with the required permits and ethical clearance, were evaluated in this study (Table 2.1). The samples, which were all bi-products from prior studies and comprised of 86 *Bathyergus suillus*, 53 *Georychus capensis*, 45 *Cryptomys hottentotus hottentotus* and 50 *Fukomys damarensis*. DNA extracts prepared using a commercial DNA extraction kit (Roche High Pure DNA extraction kit, Roche Diagnostics GmbH, Mannheim, Germany) and the manufacturer's prescribed protocol for mammalian tissue were evaluated by broad-range PCR amplification with universal 16S rRNA bacterial primers 27F and 1492R (primer set A in Table 2.2; Retief 2014). Products of the expected size (~1.4 kbp) were purified and cycle sequenced using the Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California), respectively.

Table 2.1: Weight, distribution, soil preference, digging behaviour and sampling information of the four bathyergids species screened for *Bacillus* genome presence

	Bathyergus suillus	*Georychus capensis*	*Cryptomys hottentotus hottentotus*	*Fukomys damarensis*
	Bathyergus suillus	*Georychus capensis*	*Cryptomys hottentotus hottentotus*	*Fukomys damarensis*
Average weight	504-1291 g	44-287 g	27-84 g	131 g
Distribution	Endemic to Cape floristic region	South Africa, Namibia, Botswana and Zimbabwe	Restricted to South Africa	Endemic to the western parts of southern Africa (including parts of South Africa and Zambia)
Soil preference	Sandy soils	Loam/clay soils	Loam/clay soils	Loose, arid soils
Sample size	86	53	45	50
Sample site	Cape Town airport, Western Cape	Darling, Western Cape	Darling, Western Cape (6 individuals); Kamieskroon, Northern Cape (39 individuals)	Hotazel, Northern Cape
Sampling Co-ordinates	33°58'10''S 18°35'50''E	33°25'S 18°25'E	33°25'S 18°25'E (Darling) 30°13'S 17°57'E (Kamieskroon)	27°17'S 22°58'E
Habitat type of sampling area	Peri-urban	Semi-natural	Semi-natural	Natural
Sociality	Solitary	Solitary	Social	Eusocial
Average tunnel depth	40-65 cm	3-9 cm	35 cm	21-48 cm
Deepest tunnel depth	>2m	N/A	58 cm	2.4 m
Ethics clearance	AUCC 040702-015	EC118-13	EC005-11	AO27/06

Sources: De Graaff 1981; du Toit et al. 1985; Davies and Jarvis 1986; Skinner and Smithers 1990; Jarvis and Bennett 1993; Roper et al. 2001; Bennett 2011; Robb et al. 2012; Lövy et al. 2012

2.2.2. Evaluation and optimisation of *Bacillus* assays

Bacillus genome detection capabilities of six primer sets (B-G, Table 2.2) identified through literature review were evaluated using 19 mole-rat lung extracts, confirmed to be positive for *B. cereus* group species through broad-range 16S rRNA amplification and nucleotide sequencing. These 19 positive samples served as controls for optimisation of reaction conditions for clinical specimens, which included primer concentration ranging in 0.25 µM intervals from 0.25 µM to 1 µM (Appendix 2.1) and annealing temperature ranging (Appendix 2.2) for the two group-specific primer sets (B and C) and the four type-specific *gyr*B primer sets (D-F) detailed in Table 2.2.

Table 2.2: Summary of primers and the optimised reaction conditions determined in this study

Reaction code	Primer set used (from 5' to 3')	Genome region targeted	Reference	Primer concentration used	Expected amplicon size (bp)	Ta used in study
A[1]	27F: AGA GTT TGA TCC TGG CTC AG (F) 1492R: GGC TAC CTT GTT ACG ACT T (R)	16S rRNA	Edwards et al. 1989 Reysenbach et al. 1992	0.4 µM	~1450	56ºC
B[2]	BSU063330_F: TTT ACC AGA AGC HCA GCT GC (F) BSU06330_R: GCT AAA AAT TTA ACA TCG TCT GGA (R)	*yea*C	Ahmod et al. 2011 Ahmod et al. 2011	0.4 µM	282-354	55ºC
C[3]	BCGSH-1F: GTG CGA ACC CAA TGG GTC TTC (F) BCGSH-1R: CCT TGT TGT ACC ACT TGC TC (R)	*gro*EL	Park et al. 2007 Park et al. 2007	0.5 µM	400	63ºC
D[4]	BASH-2F: GGT AGA TTA GCA GAT TGC TCT TCA AAA GA (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	253	63ºC
E[4]	BCJH-F: TCA TGA AGA GCC TGT GTA CG (F) BCJH-1R: CGA CGT GTC AAT TCA CGC GC (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	475	63ºC
F[5]	BTJH-1F: GCT TAC CAG GGA AAT TGG CAG (F) BTJH-R: ATC AAC GTC GGC GTC GG (R)	*gyr*B	Park et al. 2007 Park et al. 2007	1 µM	299	63ºC
G	BMSH-F: TTT TAA GAC TGC TCT AAC ACG TGT AAT (F) BMSH-R: TTC AAT AGC AAA ATC CCC ACC AAT (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	604	63ºC
H_1	BsGroEL-F1: CAA GTA GCT GCT ATT TCT GCA (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gro*EL	This study Park et al. 2007	0.4 µM	~260	62ºC
H_2	BsGroEL-F2: CAA GTA GCT GCT ATT TCT TCG (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gro*EL	This study Park et al. 2007	0.4 µM	~260	62ºC

T_a: Annealing temperature, [1] Amplifies broad range of bacterial species; [2] Preferentially amplifies *B. cereus* group strains (~350bp), but also targets host genome; [3] Preferentially amplifies *B. cereus* group strains (~400bp), but can result in non-specific amplification; [4] Failed to amplify *B. cereus* group strains in bathyergids; [5] Produces multiple amplicon bands either forming part of host genome or resulting in mixed sequences.

2.2.3. PCR screening and genetic characterisation

All 234 samples were screened using the optimised reaction conditions for the two group-specific *gro*EL (primer set C, Park et al. 2007) and *yea*C (primer set B, Ahmod et al. 2011) PCR assays. Amplification of both group-specific assays confirmed *B. cereus* complex presence, and samples for which conflicting results were obtained were subjected to another round of screening with both primer sets. In this way, two independent amplifications of either one, or both group-specific targets in combination with nucleotide sequencing was used to confirm *Bacillus* presence, and allowed for unequivocal assignment of a positive status.

All positive samples were subsequently screened using four species-specific primer sets (D-G) that are reported to be specific for strains of *B. anthracis* (D), *B. cereus* (E), *B. thuringiensis* (F) and *B. mycoides* (G) (Table 2.2; Park et al. 2007). Nucleotide sequences generated using species-specific primer sets were evaluated for their ability to classify *Bacillus* strains to species level.

Finally, as most of the 42 positive samples appeared to be co-infected, two primer assays (H_1 and H_2), that target the *gro*EL gene region, were newly designed to confirm the presence of each of the two *Bacillus* strains identified in the current study, and to assess levels of co-infection.

2.2.4. PCR amplification and purification

All PCRs were run on an ABI 2720 thermal cycler (Applied Biosystems, Foster City, California; Table 2.2) in a final reaction volume of 40 µl, containing 1 x Dream*Taq* Buffer, 0.2 µM dNTPs (Fermentas), 1.5 U of Dream *Taq* (Thermo Fisher Scientific, USA) and primers at reaction-specific concentrations (summarised in Table 2.2), in the presence of 3 µl of template DNA.

Touchdown PCRs with an initial denaturation at 96°C for 12 s, annealing at determined optimal annealing temperatures (Table 2.2: Appendix 2.2.) for 30 s, elongation at 70°C (with time dependent on the size of amplicon targeted) and a final elongation at 70°C for 1min, were performed for all primer sets. PCR product sizes were estimated by 1.5% agarose gel electrophoresis against a DNA molecular weight marker (Fermentas, Waltham, Massachusetts, USA). Products of the correct size were purified either directly from the tube or by gel-slice

purification using the Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.2.5. Nucleotide sequencing and phylogenetic analysis

Purified DNA was cycle sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California) with each of the external PCR primers. Sequence chromatograms viewed in the Chromas program in MEGA 6, were edited and sense and antisense strands were aligned to generate sequence contigs. The resulting sequences were used in nucleotide BLAST (Altschul et al. 1990) searches against GenBank (www.ncbi.nlm.nih.gov/blast) 33in order to identify the closest sequence matches in this public database.

The 16S rRNA, *gro*EL and *yea*C sequences generated in this study and submitted to GenBank were complemented with reference sequence data and aligned with ClustalW in Mega 6 (Tamura et al. 2013). *Paenibacillus mucilaginosus*, a sister taxon to the genus *Bacillus*, was included for outgroup purposes. Each of the three gene regions were initially individually analysed using uncorrected p-distances and the Neighbor-Joining algorithm (Saitou & Nei 1987) in Mega 6 (Figure 2.1). Individual datasets were subsequently combined to form a concatenated dataset, 2066 nt in length and comprising of 21 taxa (Figure 2.2). This 21 taxon dataset was used to identify the best-fit model of sequence evolution under the Bayesian Information Criterion (BIC) in Mega 6 (Tamura et al. 2013). Minimum Evolution (ME) and Maximum Likelihood (ML) analyses were performed in Mega6 (Nei and Kumar 2000; Tamura et al. 2013) and PhyML version 3.1 (Guindon et al. 2010), respectively. Bayesian Inference (BI) performed in MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was stopped after 1 000 000 generations. Trees were sampled every 10^{th} iteration and MCMC Trace Analysis Tool version 1.6.0 (Rambaut et al. 2014) was used to confirm a 25% burn in. Two independent MCMC runs, with one cold and three heated chains, were performed.

Differences in *Bacillus* prevalence between host species, sexes and level of urbanisation of the sampling locality (Table 2.1) were analysed using Pearson Chi-square tests in IBM SPSS (Version 23.0. Armonk, NY: IBM Corp.). All statistical analyses were performed on a sample size of 234.

2.2.6 Specificity and sensitivity calculations

Samples were only taken as being positive, if they produced sequences of the expected amplicon size. To calculate the sensitivity of a primer set the true positives (TP) were divided by the sum of the true positives (TP) and the false negatives (FN) so the equation would read as follows: TP/(TP+FN). To calculate the specificity of a primer set the true negatives (TN) were divided by the sum of the true negatives (TN) and the false positives (FP) so the equation would read as follows: TN/(TN+FP) (Altman & Bland 1994).

2.3.Results

2.3.1. Broad-range PCR confirmation of *Bacillus* genome presence

Screening of lung samples with broad range 16S rRNA primers (primer set A, Table 2.2) produced an amplicon of the expected size in 194 (83%) samples. A subset of these (*n*=73) selected for purification and nucleotide sequencing produced unambiguous sequences. Nucleotide BLAST searches revealed high levels of bacterial diversity, with *Bacillus* being the most prevalent genus, occurring in 19 of the 73 (26%) samples sequenced (Retief 2014).

2.3.2. Evaluation of *Bacillus cereus* group- and species-specific primer sets

Screening of the 234 samples with group-specific assays (B and C) revealed that *B. cereus* group strains were present in 39 of the *B. suillus* samples, two of the *C. h. hottentotus* samples and one of the *F. damarensis* samples, and absent from all 53 *G. capensis* samples (Figure 2.3). The number of sequence-confirmed *Bacillus* positives obtained with this two-gene screening approach was 42, corresponding to an overall bathyergid *Bacillus* prevalence of 17.95%. This value of 42 represented the number of true positives, guiding subsequent sensitivity and specificity calculations of the individual group-specific PCR assays.

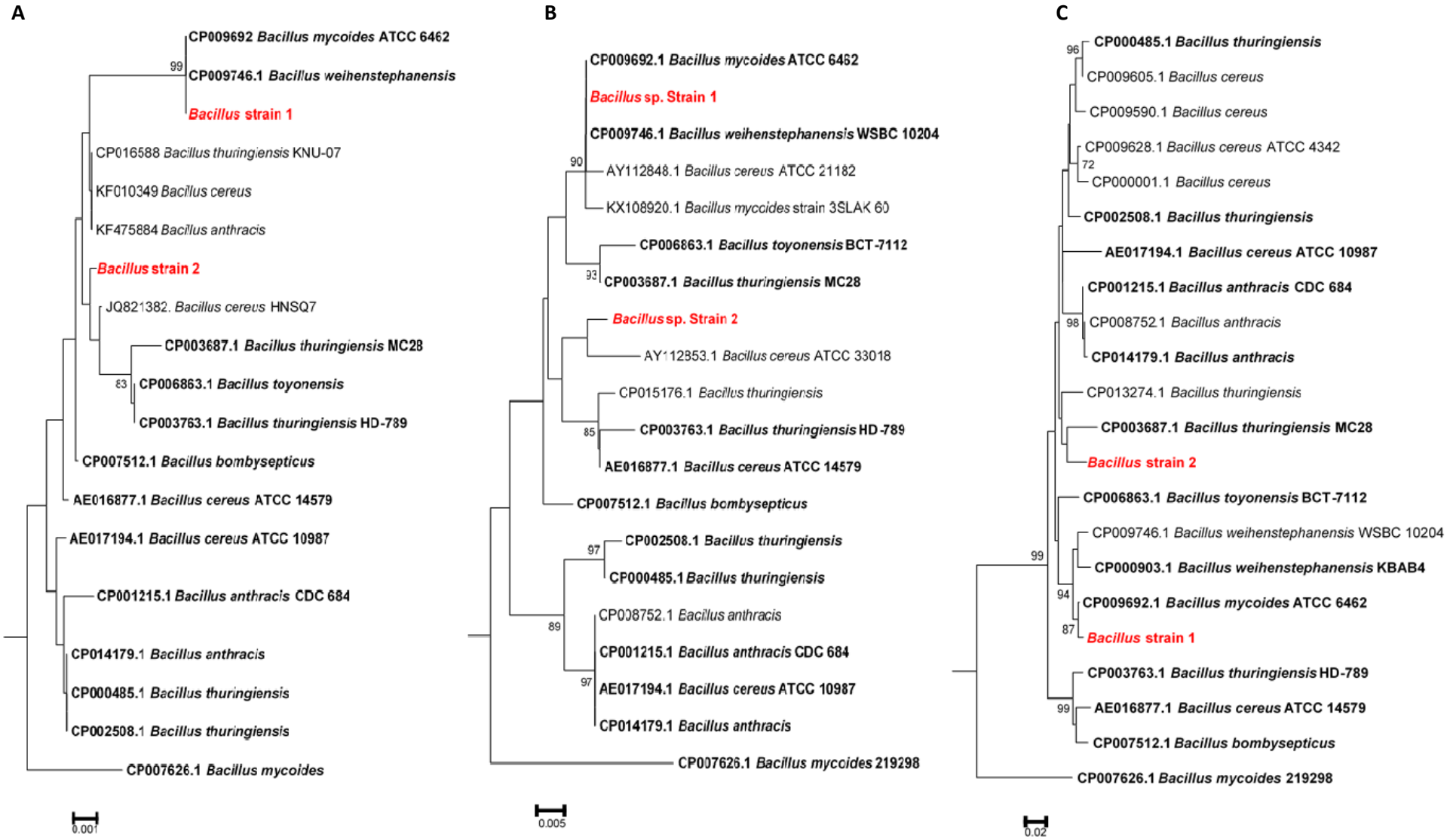

Figure 2.1: Individual p-distance Neighbour-joining gene trees indicating the *Bacillus cereus* complex subtree, constructed using an aligned 1437 nt 26 taxon dataset of the 16S rRNA gene region (A), an aligned 306 nt 26 taxon dataset of the *gro*EL gene region (B) and an aligned 312nt 26 taxon dataset of the *yea*C gene region (C). Strains found to be present in bathyergids are indicated in red, reference strains corresponding to the concatenated gene tree are indicated in bold and remaining reference strains represent other close matches to strain 1 and strain 2 obtained from GenBank through blastn searches. Bootstrap support values > 70% (obtained from 10000 bootstrap replications of each dataset are indicated next to relevant nodes of each gene tree.

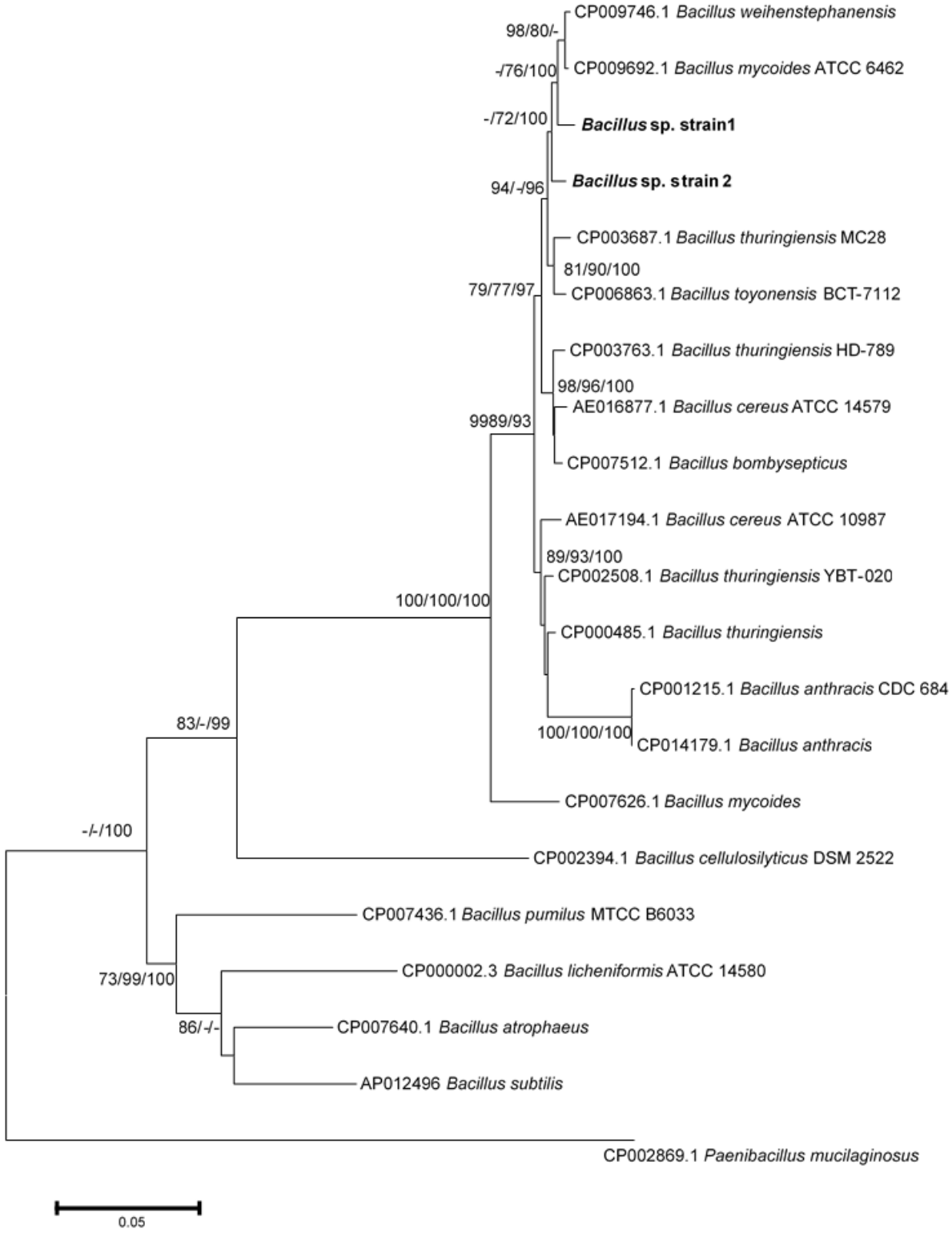

Figure 2.2: Minimum evolution tree depicting *Bacillus cereus* complex species detected in four bathyergid species (*Bathyergus suillus*, *Georychus capensis*, *Cryptomys hottentotus hottentotus* and *Fukomys damarensis*) sampled in the western Cape, South Africa (indicated in bold) with relevant reference sequences, inferred using the combined and aligned 2066 nucleotide fragment of the 16S rRNA, *gro*EL and *yea*C gene regions. Bootstrap support values ≥ 70% from the Minimum evolution analysis (ME; 10 000 bootstrap replicates), and Maximum likelihood analysis (ML; 1000 bootstrap replicates); and posterior support values ≥ 90% from the Bayesian inference (BI; 1 000 000 generations sampled every 10 generations with a 25% burn-in) are indicated as ML/ME/BI next to each node.

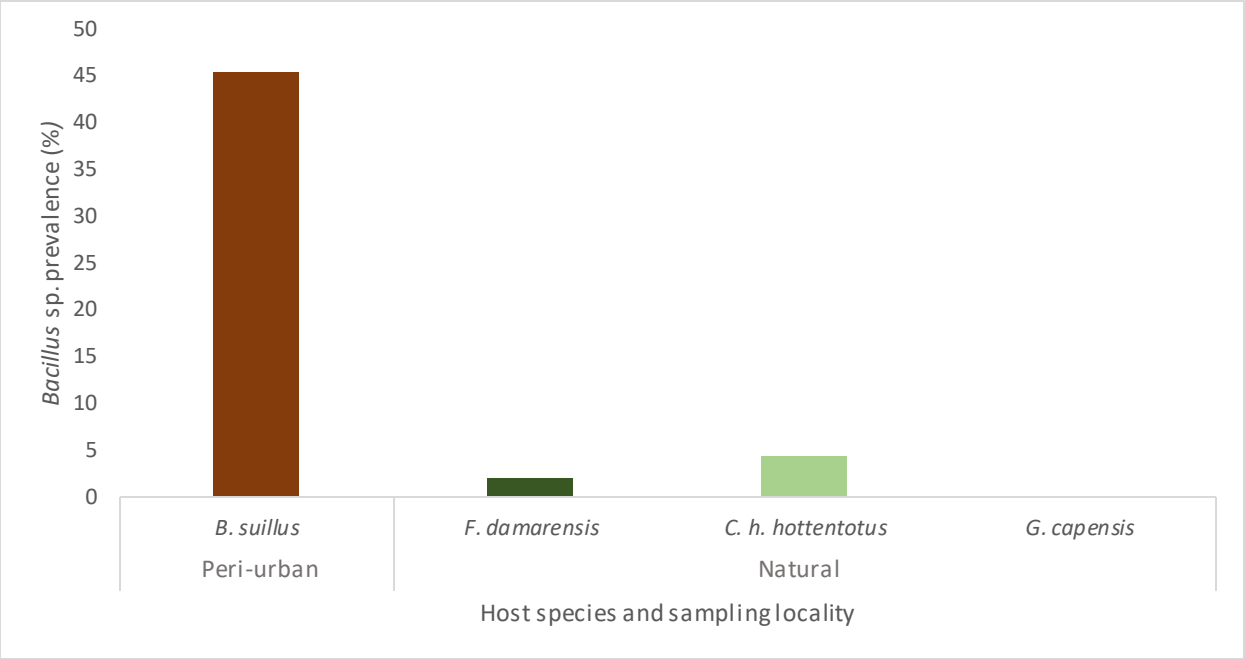

Figure 2.3: Percentage of *Bacillus* strains found to be present in the lung tissues of four bathyergid species. Bathyergid species are indicated below bars (*B. suillus* = *Bathyergus suillus*, *G. capensis* = *Georychus capensis*, *C. h. hottentotus* = *Cryptomys hottentotus hottentotus* and *F. damarensis* = *Fukomys damarensis*). A significant difference in *Bacillus* prevalence was found between *B. suillus* and the three other bathyergid species assessed.

Forty of the 234 samples were found to be positive with the *yea*C gene assay (primer set B). Of these positive samples, 33 had an expected amplicon size of ~350 bp, corresponding to an overall PCR prevalence of 14.10%. Three samples had amplicon sizes of ~200 bp, corresponding to a host genome target while the remaining four samples had an amplicon size of ~1000 bp. The calculated sensitivity and specificity values for this primer set were 78.57% and 100% respectively. With primer set C, targeting the *gro*EL gene region, 40 samples were PCR-positive. Sequencing revealed that all 40 were positive for *B. cereus* complex presence, indicating an overall PCR prevalence of 17.09%. Based on these results, primer set C had a sensitivity value of 95.24%, and a specificity value of 100%.

All 42 samples confirmed positive for *B. cereus* group by first round genus-specific PCR and nucleotide sequencing, were subjected to species-specific amplification with four primer assays (D – G), targeting the *gyr*B gene region, designed by Park et al. (2007), for which reaction conditions were optimised in this study (Table 2.2). Primer sets D and E, reported to be specific to *B. anthracis* and *B. cereus*, respectively, were negative across all samples, whereas primer set F (specific for *B. thuringiensis*) resulted in multiple bands of the incorrect size, producing either mixed or host genome sequences (Supplementary Table 2.2). Seven (16%) of the 42 samples

screened using the *B. mycoides* specific primer set (G) were positive and nucleotide sequencing of the expected ~ 600 bp amplicon confirmed that all were *B. cereus* complex strains. Two novel strains belonging to the *B. cereus* complex were identified in the current study (see 2.3.3. Nucleotide sequence analyses section) and strain-specific PCR assays (H_1 and H_2) were developed to accurately distinguish between these two strains. Additional screening with strain-specific *gro*EL primer assays H_1 and H_2 revealed high levels of co-infections (71.42% of all positive samples; 74.36% of positive *B. suillus* samples; 100% of positive *C. h. hottentotus* samples and 100% of all positive *F. damarensis* samples), and nucleotide sequencing of the products confirmed that the assays were capable of selectively amplifying each of the targeted strains (Figure 2.4), making it possible to identify with confidence the number of co-infected samples (Figure 2.5).

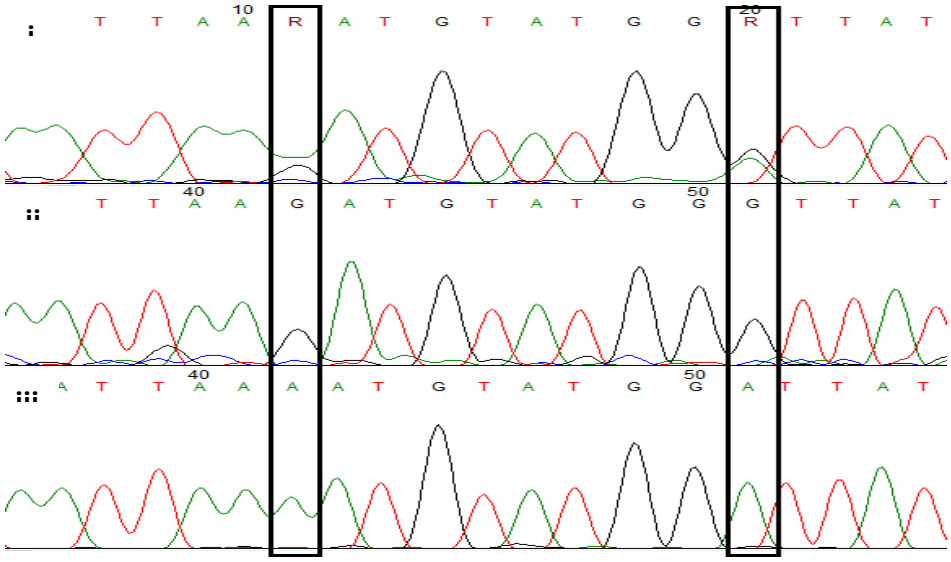

Figure 2.4: Partial chromatograms of *gro*EL sequences, sequenced using primer set C (i), primer set H_1 (ii) and primer set H_2 (iii), confirming the capability of primer sets H_1 and H_2 to selectively amplify each of the two *Bacillus* strains present within a co-infected bathyergid sample.

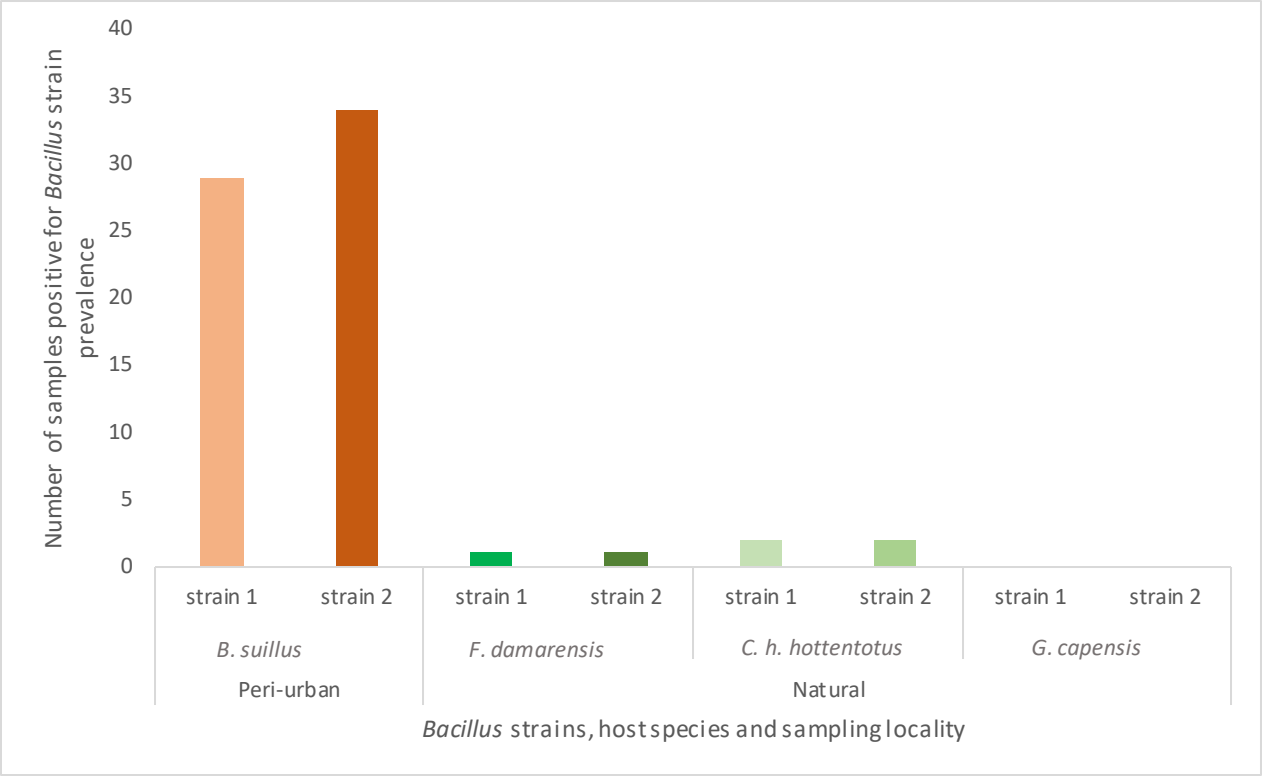

Figure 2.5: Number of samples found to be positive for the two identified *Bacillus* strains by screening with primer set H_1 and H_2 respectively per sampling locality and host species.

2.3.3. Nucleotide sequence analyses

Nucleotide sequencing and blastn results revealed that the *Bacillus* strains present in bathyergids from the Western Cape Province had high sequence identity to valid *B. cereus* complex species (Figure 2.1), for all gene regions evaluated. Sequencing of 16S rRNA, *yea*C and *gro*EL amplicons confirmed the presence of two distinct strains and the presence of mixed infections in bathyergids. *Bacillus* sp. strain 1 in bathyergids was identical to *B. mycoides* (CP009746) and *B. weihenstephanensis* (CP009746), across the *gro*EL and 16S rRNA gene regions, but differed from these reference strains by ≥3% across *yea*C (Figure 2.1). In contrast, *Bacillus* sp. strain 2 was distinct from all *Bacillus* sequence entries presently in the GenBank database, with minimum nucleotide pairwise p-distances ranging from 1% for 16S rRNA to a maximum of 4% for *yea*C (Figure 2.1). The phylogenies inferred using the concatenated dataset of the 16S rRNA, *gro*EL and *yea*C gene regions (Figure 2.2), revealed that the *Bacillus* strains identified in bathyergids clustered within a well-supported *B. cereus* complex clade. In common with previous reports (Helgason et al. 2000; Guinebretière et al. 2008; Tourasse et al. 2011), the phylogenies confirmed that valid members of recognised species did not form monophyletic lineages, but were intermixed with one another (Figures 2.1 and 2.2).

2.3.4 Statistical analyses

A significant difference in *Bacillus* occurrence was found between different host species (χ^2 = 69.643; df = 3; $p < 0.05$; Figure 2.4) and between different sampling localities (χ^2 = 70.245; df = 3; $p < 0.05$), while no significant difference was found between different host sexes (χ^2 = 0.741; df = 1; p = 0.389).

2.4. Discussion

Continuous human encroachment into natural environments has led to an increased risk of people coming in contact with animals and environments harbouring emerging and re-emerging bacterial pathogens (Comer 2001). Increased human susceptibility, due to diseases such as HIV/AIDS and cystic fibrosis, has amplified the need to understand disease dynamics of opportunistic pathogens (Berg et al. 2005). Therefore, rapid and accurate methods are needed to identify etiological agents present in previously unidentified or overlooked environments and hosts (Shabbir et al. 2015). Due to their subterranean lifestyle, and high-level contact with soil and the rhizosphere, bathyergid species hold potential as sentinels for opportunistic soil-dwelling bacterial pathogens.

The observation that all *Bacillus* strains detected in bathyergid lungs, formed part of the *B. cereus* complex (Figures 2.1 and 2.2), supports the view that bathyergids may act as reservoirs of potential soil-borne pathogens. Of concern is the significantly higher prevalence of *B. cereus* species complex strains in *B. suillus* lungs, compared to the three other bathyergid species assessed (Figure 2.3). Anthropogenic activities, such as ploughing or the use of manure for agriculture, the runoff of wastewater into ecosystems, or the use of microbes for bio-control or bio-remediation can alter the community structure of soil microbiota, increasing opportunistic pathogen presence in soil systems (Berg et al. 2005; Jackson et al. 2009; Aujoulat et al. 2012; Paulse et al. 2012; Duran-Alvarez and Jimenez-Cisneros 2014). Therefore, the high *Bacillus* spp. prevalence found in individuals of *B. suillus*, sampled in a peri-urban setting, compared to that of its relatives, sampled in natural settings where bathyergid-microbe interactions are considered ancient, may be attributed to anthropogenic involvement, and emphasises the potential of *B. suillus* to act as a sentinel for anthropogenic soiling.

Both *Bacillus* species and bathyergid species have a close association with specific soil conditions. Therefore, it could be argued that sampling hosts with different burrowing behaviour

in different soil and climatic conditions may have influenced the observed *Bacillus* prevalence in the host species assessed. However, Brillard et al. (2015) found that, while *B. cereus* complex species most likely only grow vegetatively within the rhizosphere, *B. cereus* complex spores show no difference in abundance at different soil depths, suggesting that disparate burrowing behaviour displayed by the bathyergid species (Table 2.1), only plays a limited role in the observed differences in the occurrence of *B. cereus* complex strains within the lungs of these host species.

Possibly of greater importance is the difference in prevailing climatic conditions of the distributional areas of bathyergids along with the structure of soils inhabited by *B. suillus* and its relatives. However, a study performed on soil samples obtained from Thailand, Munich (Germany) and the Karwendel mountains (Austria), revealed that species of the *Bacillus cereus* complex have higher diversity in temperate climates, high in temperature fluctuations, compared to those occurring in climates with extreme temperatures, showing low fluctuations in mean temperature (Von Stetten et al. 1999). The *B. cereus* complex thus consists of both mesophilic species (with a growth range from above 7˚C to 46˚C) and psychrotolerant species (with a growth range from below 7˚C to 38˚C), with high fluctuation of average temperatures favouring selection for more than one thermal type (Von Stetten et al. 1999; Guinebretière et al. 2010). Thus, although the bathyergid species occur across a broad range of rainfall areas (Table 2.1), the temperate environment inhabited by *B. suillus* would likely only have influenced the number of *B. cereus* complex strains that individuals were exposed to.

In terms of soils inhabited, *B. suillus* and *F. damarensis* prefer sandy soils that are less compact compared to the loam clay soils preferred by the both *C. h. hottentotus* and *G. capensis* (De Graaff 1981; Bennett 2011; Robb et al. 2012). Little is known about the soil preferences of *B. cereus* complex strains, although strains seemingly prefer alkaline soils, high in calcium and moisture content with high levels of organic matter (Dragon and Rennie 1995; Smith et al. 1999; Jensen et al. 2003; Vilain et al. 2006; Blackburn et al. 2014). As soil alkalinity and soil nutrient content are heavily affected by a range of factors (including anthropogenically introduced contaminants, the community structure of plants inhabiting an area and the parent material from which the soil has been weathered), further investigation is required into the effect that specific conditions of soils inhabited by bathyergids may have on their infection levels (Chesworth 1973; Sparks 1995).

The phylogenetic results confirm previously reported difficulties with *Bacillus* species identification (Helgason et al. 2000; Guinebretière et al. 2008; Tourasse et al. 2011), in that members of valid species of the *B. cereus* complex are distributed throughout the phylogeny (Figure 2.2), supporting the suggestion that some recognised species within this bacterial group do not constitute monophyletic lineages (Zwick et al. 2012). This is likely due to the high degree of similarity displayed between recognised species, highlighting the difficulty in conducting genetic studies on the *B. cereus* complex.

The current study confirmed the value of the Park et al. (2007) *gro*EL PCR assay for detecting *B. cereus* complex species, as this assay showed high levels of sensitivity and specificity, relative to the combined two-gene approach against which it was benchmarked. It also emphasised the need to identify alternative primer sets that are better suited to assessing bacterial diversity in small mammals such as bathyergids, as host genome amplification, that occurred with primer sets B and F, targeting *yea*C and *gyr*B, respectively (Table 2.2), can lead to false positive results. The value of novel test development was demonstrated by the newly-developed PCR assays which selectively amplified each of the *Bacillus* lineages identified in this study, allowing for the determination of levels of co-infection, and confirming the higher levels of strain diversity in the temperate environment occupied by *B. suillus* (Figure 2.5).

Belowground biodiversity is severely understudied, with the human health implications of interactions between different classes of soil biota, such as microbe-bathyergid interactions, generally being overlooked (Berg et al. 2005; Parker 2010). With continual anthropogenic pollution of once natural environments worldwide, the risk of introducing opportunistic pathogens into transformed ecosystems, particularly in developing countries such as South Africa, has increased (Berg et al. 2005; Paulse et al. 2012; Duran-Alvarez and Jimenez-Cisneros 2014). This, together with climate change, is expected to increase emergent disease incidence, emphasising the need to establish suitable methods for identifying potential reservoirs and routes of transmission (McMichael et al. 2006; Gale et al. 2009; Carlin et al. 2010). Consequently, the 45.35% prevalence of *Bacillus cereus* complex strains in *B. suillus* is significant as this bathyergid species occurs near informal human settlements in which the proportion of immune-compromised individuals is high.

Conclusion

The findings of this study highlight the need for additional research into the roles of anthropogenic pollution, climatic factors and soil structure, on the maintenance and community structure of potentially pathogenic soil microbiota within the environment. It also underscores the value of soil-dwelling mammals, as early detection systems for opporunistic pathogens, and as indicators of environmental soiling.

Chapter 3

Detection of novel haemoplasma strains in four species of free-living bathyergids from the Western Cape Province, South Africa

Abstract

Haemotropic *Mycoplasma* species (haemoplasmas) are unculturable obligate erythrocytes-infecting bacteria of zoonotic concern, and represent a group of bacterial pathogens for which data on prevalence and diversity in wildlife is limited. Members of the subterranean rodent family Bathyergidae, vary in their level of exposure to human-transformed landscapes, with one species, *Bathyergus suillus*, often captured and consumed by poorer communities, facilitating the spread of potential zoonotic pathogens. The current study assessed haemoplasma prevalence and diversity in four bathyergid species whilst simultaneously optimising 16S rRNA PCR assays for improved detection of haemoplasma strains. Bathyergid lung, spleen and liver samples were screened using one published and two novel PCR assays all targeting the 16S rRNA gene region. Of the 286 samples screened, an overall haemoplasma prevalence of 24.13% was found, with *B. suillus* having a significantly higher prevalence (41.86%) and diversity (six novel strains were identified) of haemoplasma compared to its close relatives for which prevalence ranged from 0%-36% prevalence and strain diversity from zero to three strains. Novel PCR assays had both higher sensitivity and specificity than the published PCR assay and phylogenetic analyses revealed that the six novel strains identified in the current study grouped well within their own cluster. Factors such as the human introduction of commensal rodents harbouring *Mycoplasma* strains and likely transferred through cosmopolitan arthropod vectors to indigenous bathyergids, as well as biotic and abiotic factors affecting ectoparasite load could have influenced haemoplasma prevalence. The PCR assay developed in this study holds potential for improving our knowledge of haemoplasma strain distribution and diversity in indigenous rodent populations.

Key words: *Mycoplasma*, haemotropic, bathyergids, arthropod-transmitted disease, South Africa, indigenous rodents

3.1. Introduction

Haemotropic *Mycoplasma* species (haemoplasmas) are gram-positive, unculturable, obligate erythrocyte pathogens (Messick 2004). In recent years, haemoplasmas have garnered interest worldwide since being linked to infections ranging from acute haemolysis (resulting in symptoms such as anorexia, lethargy, dehydration, weight loss, pyrexia and even death), to asymptomatic, with the latter suggesting that healthy hosts act as carriers of these pathogens (Willi et al. 2007b). Haemoplasmas infect a wide range of mammalian hosts and, although haemoplasma strains are generally considered to be host-specific, there is some evidence of interspecies transfer (Willi et al. 2007a; Maggi et al. 2013a), and zoonotic potential is increasingly recognised (Hu et al. 2004; Neimark et al. 2005; Sykes 2010; Maggi et al. 2013b). Blood-sucking arthropods (lice, fleas, ticks and mites) have been implicated as primary vectors for haemoplasma strains, and as immune-deficiency severely increases susceptibility to infection, immune-compromised patients with increased exposure to blood-sucking arthropods are considered to be at higher risk of haemoplasma infection and resulting haemolysis (Willi et al. 2006; Gonçalves et al. 2015).

The inability to cultivate these bacterial strains has resulted in a limited understanding of both the biology and pathogenesis of these organisms and, as a consequence, classification of haemoplasma strains is reliant on nucleotide sequences obtained following genomic amplification achieved with various PCR assays (Messick 2004; Sashida et al. 2013). Microscopic evaluations of blood smears have proven too insensitive, and other genetic techniques, such as DNA:DNA hybridization which require large amounts of DNA are not possible as culturing is a pre-requisite (Tasker et al. 2003; Conrado et al. 2015). Accordingly, PCR-sequencing approaches are currently seen as the gold standard for accurately detecting and identifying haemoplasma strains present in different hosts, with 16S rRNA gene sequences forming the bulk of the genetic data currently available for different haemoplasma strains (Conrado et al. 2015).

Blood-sucking arthropod-borne diseases place severe burdens on developing countries, such as South Africa (Hill et al. 2005) and, although the first haemoplasma strain (then classified as *Eperythrozoon felis*) was detected in 1942 in an anaemic cat from South Africa (Sykes 2010), data on haemoplasma strains present in wildlife from South Africa are limited. Moreover, phylogenetic analyses have shown that "*Candidatus* Mycoplasma turicensis", a species associated with haemolytic anaemia in domesticated and wild cats, and previously detected in domesticated cats sampled in Johannesburg, South Africa (Willi et al. 2006), is more closely related to *M.*

haemomuris and *M. coccoides*, two rodent-associated haemoplasma strains, than it is to other haemoplasmas infecting felids (Willi et al. 2007a). This discovery has led to the hypothesis that rodent-borne haemoplasmas may be capable of interspecies transmission to other mammalian hosts (Willi et al. 2007a; Maggi et al. 2013a).

The prevalence of haemoplasmas has been well documented in domestic animals, but large gaps still remain in understanding their prevalence, diversity, distribution and mode of transmission as well as the effects of haemoplasmas on the health of wildlife (Gonçalves et al. 2015). Members belonging to the order Rodentia, the most diverse and widespread order of all mammals, have been identified as possible carriers of haemoplasmas (Gonçalves et al. 2015; Hornok et al. 2015). However, the zoonotic potential of these rodent-infecting haemoplasmas has not been established, and the prevalence of haemoplasmas in wild rodent populations remains severely under-documented (Sashida et al. 2013). It is clear from recent reports of novel strains in rodent species from diverse localities (Vieira et al. 2009; Sashida et al. 2013; Hornok et al. 2015) that further investigation into haemoplasma strain dynamics in other wild rodent populations is required. Moreover, given that relatively few studies have focused on potential pathogens within indigenous rodent communities in South Africa (Taylor et al. 2008), looking at haemoplasma prevalence and diversity in native South African rodent species is of relevance.

In South Africa, up to 30% of people living in and around Cape Town are HIV-positive (Kenyon et al. 2013), significantly increasing their risk of contracting acute haemolysis should they be infected with zoonotic haemoplasma strains. Communities with the highest HIV prevalence are concentrated in resource-poor, informal settlements, often relying on alternative protein sources, including the Cape dune mole-rat (*Bathyergus suillus*) to supplement their diet. This mole-rat species, along with its co-occurring relatives *C. h. hottentotus* and *G. capenis* and close relative *F. damarensis*, belong to the family Bathyergidae, a subterranean family of African rodents with varying levels of sociality (Table 3.1). Haemoplasma strains have previously been identified in captive bathyergid colonies that succumbed to infection (unpublished results; Bastos, Lutermann & Bennett 2013), however, the potential of wild populations of bathyergids to harbour opportunistic bacterial pathogens is not known. Consequently, the current study aimed to assess both haemoplasma prevalence and strain diversity in wild-caught and captive-held bathyergid species, whilst simultaneously optimising 16S rRNA PCR assays for improved detection of haemoplasma strains in environmental and clinical samples.

3.2. Methods

3.2.1. Study sites and collection

Lung, liver and spleen samples from members belonging to the family Bathyergidae were evaluated in this study (Table 3.1). No animals sampled were represented by more than one tissue sample. Wild-caught *Fukomys damarensis, C. h. hottentotus* and *Georychus capensis* individuals were trapped using Hickman traps while wild-caught *Bathyergus suillus* individuals were captured using modified snap traps. Both wild-caught and captive-held animals were euthanised by halothane overdose as prescribed by the ethics committee of the University of Pretoria. DNA extracted from five flea samples belonging to the family Hystrichopsyllidae and two lice samples, collected from *B. suillus* were also made available. The tissue samples stored in absolute ethanol and the DNA extracted from ectoparasite samples were all bi-products from prior studies.

3.2.2. DNA extraction

DNA was extracted from lung samples of 86 *B. suillus*, 45 *C. h. hottentotus*, 53 *G. capensis* and 50 *F. damarensis* individuals; from liver samples of 28 *B. suillus* individuals, extracted during a previous study, and from spleen samples of 17 laboratory-housed *C. h. hottentotus* individuals that succumbed to infection, using a commercial DNA extraction kit (Roche High Pure DNA extraction kit, Roche Diagnostics GmbH, Mannheim, Germany). As part of a previous study, the manufacturer's prescribed protocol was used to extract DNA from the five fleas and two lice samples (Van Sandwyk 2007).

3.2.3. Y-chromosome typing

As information on the sex of five *B. suillus* and two *F. damarensis* samples were unavailable, bathyergid sex was confirmed through screening DNA extracts with published Y-chromosome primers UTY11F and UTY11R (Hellborg and Ellegren, 2003).

Table 3.1: Sampling information, distribution, sociality level, digging depth, preferred habitat type and haemoplasma prevalence estimates of the four bathyergids species screened for hemoplasma prevalence

Host species	Sample type	Sample site	Sampling co-ordinates	Anthropogenic influence of sampling areas	Distribution	Level of sociality	Average tunnel depth	Preferred habitat	Ethics clearance	Total number of samples analysed	% of confirmed haemoplasma positives	Novel Haemoplasma strains present	Number of co-infections
B. suillus	Lung	Cape Town airport, Western Cape	33°25'S 18°25'E	Urban	Endemic to Cape floristic region	Solitary	40-65 cm	Temperate areas	AUCC 040702-015	86	41.86%	6	7
	Liver									28	32.14%	2	1
	Flea				n/a					5	0%	0	0
	Lice				n/a					2	0%	0	0
F. damarensis	Lung	Hotazel, Northern Cape	27°17'S 22°58'E	Natural	Endemic to the western parts of southern Africa (including parts of South Africa and Zambia)	Eusocial	21-48 cm	Arid areas	AO27/06	50	36%	2	0
C. h. hottentotus	Lung	Darling, Western Cape	33°25'S 18°25'E	Semi-natural	Restricted to South Africa	Social	35 cm	Temperate and Arid areas	EC005-11	6	11%	3	1
		Kammieskroon, Northern Cape	30°13'S 17°57'E							39			
	Spleen	Captive-held								17	5.88%	1	
G. capensis	Lung	Darling, Western Cape		Semi-natural	South Africa, Namibia, Botswana and Zimbabwe	Solitary	3-9 cm	Temperate areas	EC118-13	53	0%	0	0
Total										286	24.13%	6	9

3.2.4. PCR assay selection and development

Extracted DNA was tested for haemoplasma strain presence using three PCR assays, all targeting the 16S rRNA gene region (Table 3.2). Firstly, a ~ 1000 nt fragment was targeted using previously described primers, Myco16S-322s and HemMycop16S-1420as (Maggi et al. 2013b), this served as PCR assay "I" (Table 3.2). For the second PCR assay (J), two additional primers, MyChlos-1F and Mycop-1R were newly designed on the basis of conserved regions identified in a complete 16S rRNA dataset compiled from reference strain sequences of valid haemotropic *Mycoplasma* species sourced from the GenBank database (www.ncbi.nlm.nih.gov). The dataset which consisted of 38 taxa was aligned and primers targeting a ~300 bp fragment within the 1273 nt in length were designed using the guidelines of A. D. S. Bastos (unpublished) (binding sites of these primers as provided in Appendix 3.1). Finally, the third PCR assay (K) consisted of the combination of a universal bacterial primer 27F with Mycop-1R, which targets a ~700 bp fragment (Table 3.2). The *Mycoplasma* strain detection capabilities of these three primer sets were evaluated using three mole-rat lung extracts, previously confirmed to be positive for haemoplasma species (Retief 2014) through broad-range 16S amplification (primer set A in Table 2.2) and nucleotide sequencing. These three positive samples served as controls for optimisation of reaction conditions for clinical specimens, which included annealing temperature ranging for the three primer sets tested (I-K), detailed in Table 3.2.

Table 3.2: Summary of PCR assays used in this study.

PCR assay	Primer set used (from 5' to 3')	Reference	Expected amplicon size (bp)	Ta used in study
I	Myco16S-322s: 5'-GCCCATATTCCTACGGGAAGCAGCAGT-3' (F)	Maggi et al. 2013b	~1000bp	68˚C
	HemMycop16S-1450as: 5'-GTT TGA CGG GCG GTG TGT ACA AGA CC-3'(R)	Maggi et al. 2013b		
J	MyChlo-1F: TGC CAG CAG CTG CGG TAA TAC (F)	This study	~300bp	69˚C
	Mycop-1R: CGT TTA CGG TGT GGA CTA CTG (R)	This study		
K	27F: AGA GTT TGA TCC TGG CTC AG (F)	Edwards et al. 1989	~700bp	61˚C
	Mycop-1R: CAG TAG TCC ACA CCG TAA ACG (R)	This study		

3.2.5. PCR cycling conditions

All three PCR assays were run on an ABI 2720 thermal cycler (Applied Biosystems, Foster City, California; Table 3.2.) in a final reaction volume of 40 µl. To ensure consistency between samples, a master mix was prepared with a final concentration of 1 x Dream*Taq* Buffer, 0.2 µM dNTPs (Fermentas), 1.5 U of Dream *Taq* (Thermo Fisher Scientific, USA), 0.4 µM of each primer, and 3 µl of template DNA was added to each tube containing 37 µl of master mix.

Touchdown PCRs with an initial denaturation at 96°C for 12 s, primer annealing at determined optimal annealing temperatures (Table 3.2) for 30 s, elongation at 70°C (with time dependent on the size of amplicon targeted) and a final elongation at 70°C for 1 min, were performed for all three primer sets. The PCR products were separated by 1.5% agarose gel electrophoresis and sized against a DNA molecular weight marker (Fermentas, Waltham, Massachusetts, USA). Products of the correct size viewed under UV irradiation were purified directly from the tube using the Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and supplier-prescribed protocols.

3.2.6. Nucleotide sequencing and phylogenetic analysis

Purified DNA was cycle sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Sequence chromatograms viewed in the Chromas program in MEGA 6 (Tamura et al. 2013), were edited and aligned to generate sequence contigs. Each of the resulting sequences was used to perform nucleotide BLAST (Altschul et al. 1990) searches against the GenBank database (www.ncbi.nlm.nih.gov/blast) in order to identify the closest sequence matches. The sequence chromatograms for each of the strains identified, was used as a reference when evaluating chromatograms confirming the presence of multiple strains in a single sample (Figure 3.1). In this way, it was possible to accurately determine the total number of co-infected samples and to identify each of the specific co-infecting strains. These results guided subsequent statistical analyses.

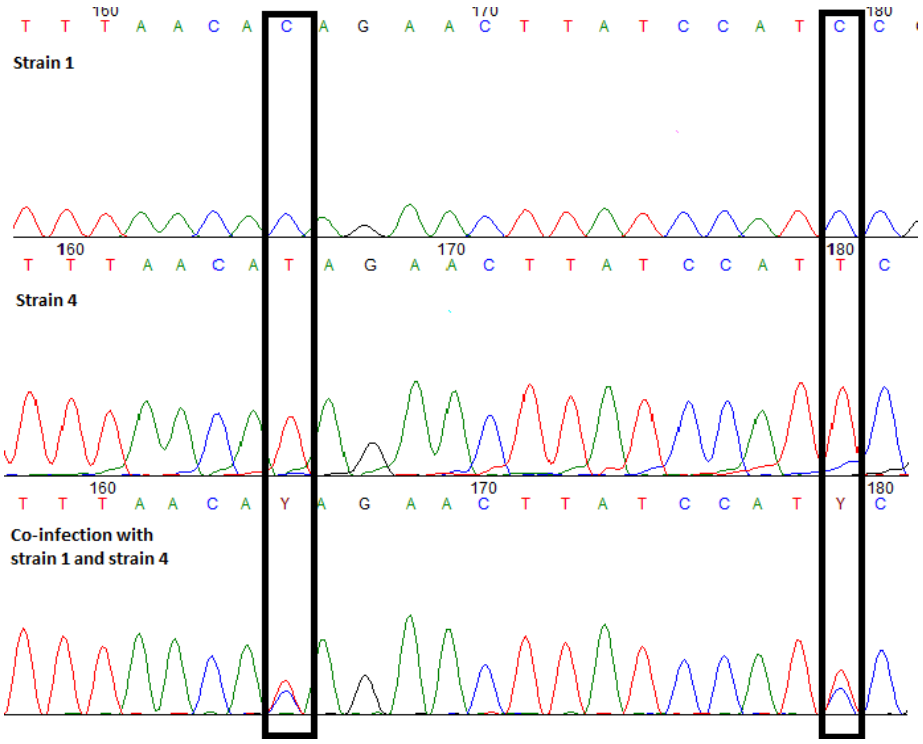

Figure 3.1: Simplified example of how strains present in each co-infected sample were identified. For each co-infected sample the entire chromatogram was assessed and compared to each of the six strains identified to reliably determine which strains were present.

Nucleotide sequences generated in this study were complemented with reference sequence data and aligned with ClustalW in Mega 6 (Tamura et al. 2013). *Mycoplasma pneumoniae* and *Mycoplasma fastidiosum*, two closely related sister taxa to the haemotropic *Mycoplasma* species lineage, were included for outgroup purposes. The final aligned 16S rRNA dataset, 625 nt in length and comprising of 34 taxa was used to compute pairwise distances between different taxa and infer a p-distance neighbor-joining tree (Saitou & Nei 1987), in Mega 6 (Tamura et al. 2013).

3.2.7. PCR assay accuracy assessment

Guided by the overall number of samples confirmed positive for *Mycoplasma* strain presence through nucleotide sequencing of all amplicons of the expected size, the relative sensitivity and specificity of each PCR assay could be calculated. To calculate the sensitivity of a primer set the true positives (TP) were divided by the sum of the true positives (TP) and the false negatives (FN) so the equation would read as follows: TP/(TP+FN). To calculate the specificity

of a primer set the true negatives (TN) were divided by the sum of the true negatives (TN) and the false positives (FP) so the equation would read as follows: TN/(TN+FP) (Altman and Bland, 1994).

3.2.8. Statistical analyses

To control for potential confounding factors, such as different haemoplasma infection rates in different host organs and differences in haemoplasma prevalence between free-roaming and captive-held bathyergids, only data obtained from lung samples of wild-caught bathyergids were included in statistical analyses. Differences in overall haemoplasma prevalence between host species, sexes and level of urbanisation of the sampling locality (Table 3.1) were analysed using Pearson Chi-square tests in IBM SPSS (Version 23.0. Armonk, NY: IBM Corp.). The differences in individual strain prevalence between host species were analysed using either Pearson Chi-square tests (for strain 4) or, as the assumption of the Pearson Chi-square test stating that the number of cells with an expected count must be less than 20% was violated in some cases, the Likelihood Ratio (for strain 1 – 3 and strain 5 – 6), both performed in IBM SPSS (Version 23.0. Armonk, NY: IBM Corp.). All statistical analyses were performed on a sample size of 234.

3.3. Results

3.3.1. Broad-range PCR confirmation of *Mycoplasma* strain prevalence

Screening of lung samples with broad range 16S rRNA primers (primer set A, Table 2.2) produced an amplicon of the expected size in 194 (83%) samples. A subset of these (*n*=73) selected for purification and nucleotide sequencing produced unambiguous sequences, of which three samples, one *B. suillus* sample (sample number 97) and two *F. damarensis* samples (sample number 106 and 107) were found to be positive for *Mycoplasma* genomic DNA, resulting in a *Mycoplasma* sp. prevalence of 1.37%.

3.3.2. *Mycoplasma* prevalence and diversity

Screening and sequencing confirmed that of the 286 (lung, liver and spleen) samples analysed, 69 samples were positive for haemoplasma strains, corresponding to an overall infection rate of 24.13% (Table 3.1; Figure 3.2). Six discrete strains/haplotypes were discernible from nucleotide sequencing, with nine samples being co-infected with more than one haemoplasma

strain (results from molecular screening are indicated in Table 3.1 and Figure 3.3), corresponding to a 15.25% co-infection rate for the positive samples.

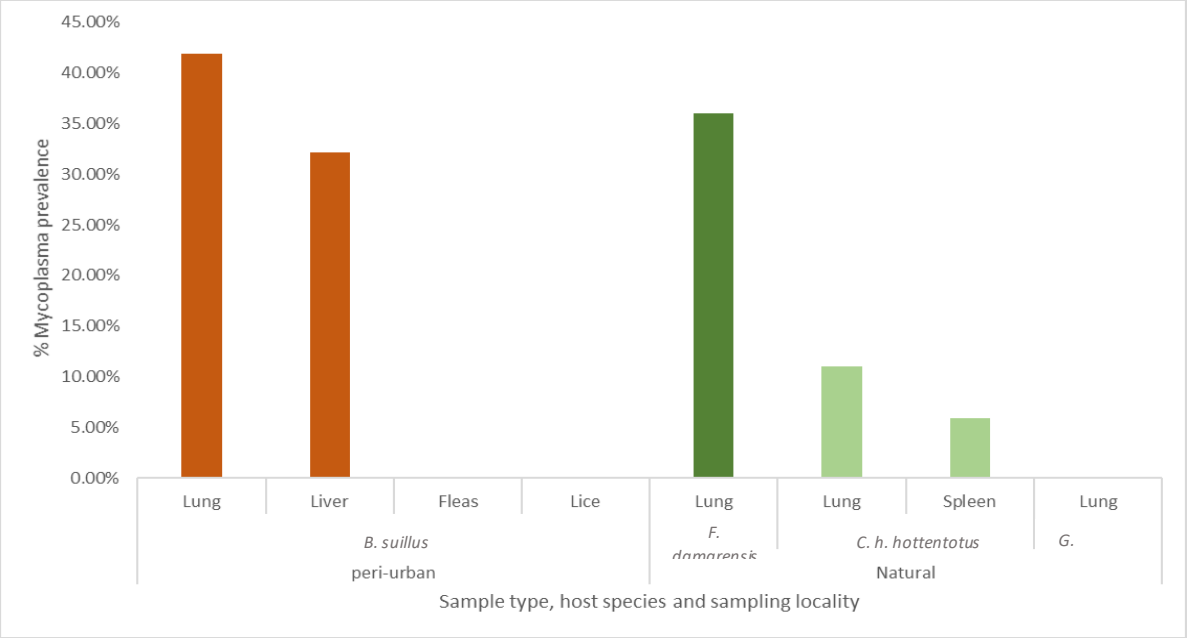

Figure 3.2: Percentage of *Mycoplasma* positive samples per sample organ for each of the four bathyergid species assessed at each of the different sampling localities.

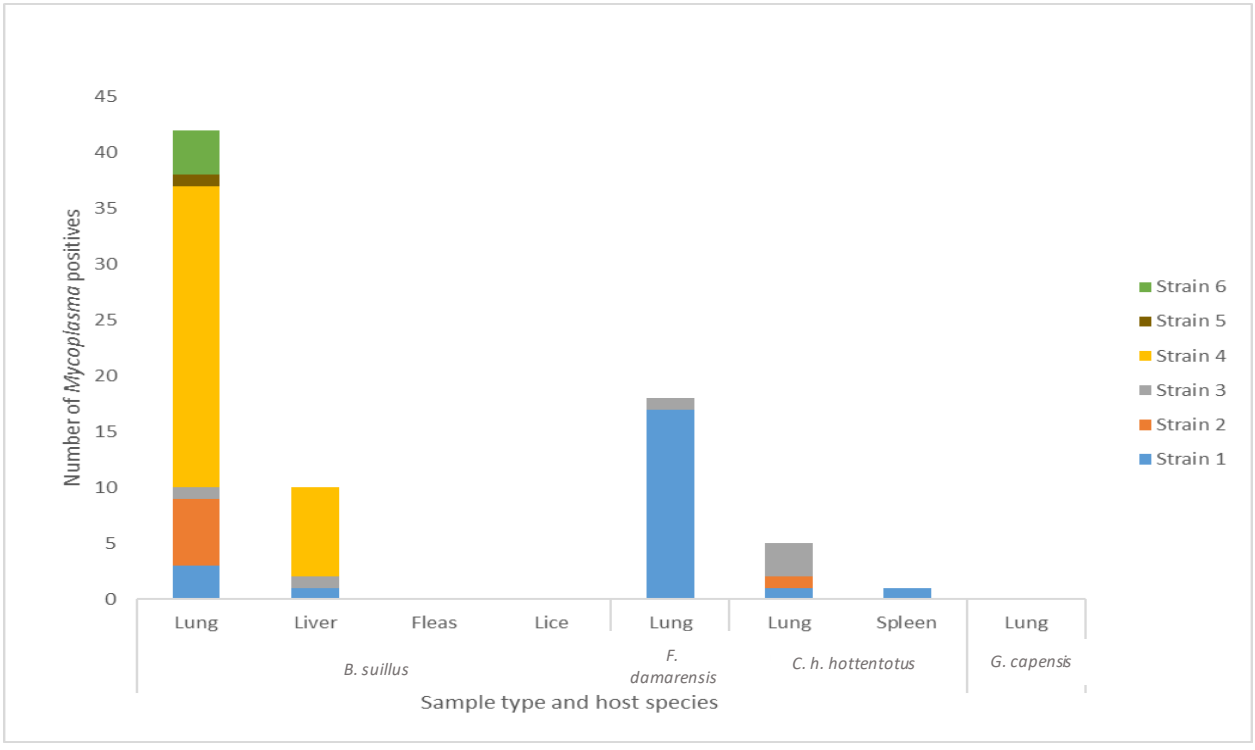

Figure 3.3: Total number of novel *Mycoplasma* strains detected in samples tested for *Mycoplasma* prevalence per sample type and host species.

3.3.3. Evaluation of the three PCR assays

Of the 286 samples screened with the published primer assay I, 52 samples were PCR positive and produced an expected amplicon size of ~1000 bp. However, sequencing and blastn searches revealed that only 42 of these samples were positive for *Mycoplasma* strains, with one sample producing a mixed sequence and strains belonging to other bacterial genera being amplified in the remaining nine positive samples (Table 3.3). This resulted in an overall prevalence of 14.68% for this primer assay, with the calculated sensitivity and specificity values being 60.87% and 95.83%, respectively.

For the newly-designed primer set J, of the 286 samples screened, 54 were PCR positive and had an expected amplicon size of ~300 bp. Sequencing and blastn searches revealed that all PCR positive samples were positive for *Mycoplasma* genomic DNA, resulting in an overall prevalence of 18.88% for primer assay K, with the sensitivity and specificity values for this assay being 78.26% and 100% respectively.

Regarding primer assay L, of the 286 samples screened, 58 were PCR positive and produced an expected amplicon size of ~700 bp. Sequencing and blastn searches revealed that of these 58 samples, 50 were positive for *Mycoplasma* genomic DNA, while one sample produced a mixed sequence and strains belonging to other bacterial genera were amplified in the remaining seven samples (Table 3.3). This resulted in an overall *Mycoplasma* prevalence of 17.48%, resulting in a calculated sensitivity of 72.46% and a specificity of 95.85%.

Table 3.3: Summary of bacterial genera other than *Mycoplasma* amplified by PCR assays used in this study.

	Bacterial genus	**Sequence identity**	**Number of samples**
Primer assay I			
	Salmonella sp.	97%	1
	Bacillus sp.	92%-99%	2
	Pseudomonas sp.	99%	3
	Clostridium sp.	99%	1
	Sporosacina sp.	92%	2
Primer assay K			
	Bacillus sp.	98%-99%	4
	Pseudomonas sp.	99%	1
	Bronchotrix sp.	99%	1
	Sporosacina sp.	99%	1

3.3.4. Nucleotide sequence analyses

Nucleotide sequencing and blastn analyses showed that all six haemoplasma strains characterised in the current study had a sequence identity of between 94%-96% to a valid *Mycoplasma coccoides* strain. Guided by the sensitivity and specificity results and, given that all novel strains recovered by PCR assay J were also represented by sequences recovered by PCR assay K, but not by sequences recovered by PCR assay I, and that PCR assay K yielded a larger DNA fragment (~700 nt) compared to PCR assay J (only ~300 nt), only those sequences generated through sequencing of the larger products (PCR assays I and K) were included in the final phylogenetic analyses.

As a 130 nt fragment on the 5' end of the 16S rRNA sequences generated in this study could not be aligned with confidence against valid haemoplasma strains obtained from GenBank, this fragment was excluded from further analyses, resulting in a final dataset 625 nt in length.

Phylogenetic analyses revealed that the six novel haemoplasma strains, present in the analysed samples, formed a well-supported, distinct cluster comprising of two discrete, well-supported sub-clusters (sub-cluster I consisting of strains 1, 2, 3 and 5 and sub-cluster II consisting of strains 4 and 6), with all six strains being sister to *Mycoplasma coccoides* (Figure 3.3). Pairwise distances revealed that the genetic distances between *M. coccoides* and novel *Mycoplasma* strains detected in the current study ranged from 4.5% to 5.2%. Pairwise distances also revealed that genetic distances between novel *Mycoplasma* strains belonging to sub-cluster I and novel *Mycoplasma* strains belonging to sub-cluster II ranged from 2.5% to 3.0%, which, is greater than the 0.8% distance (on nucleotide level), between *M. haemofelis* and *M. haemocanis*, two valid haemoplasma species. Finally, phylogenetic analyses showed that the detected strains fell within the haemofelis cluster with a high level of confidence (bootstrap values of 99%; Figure 3.3).

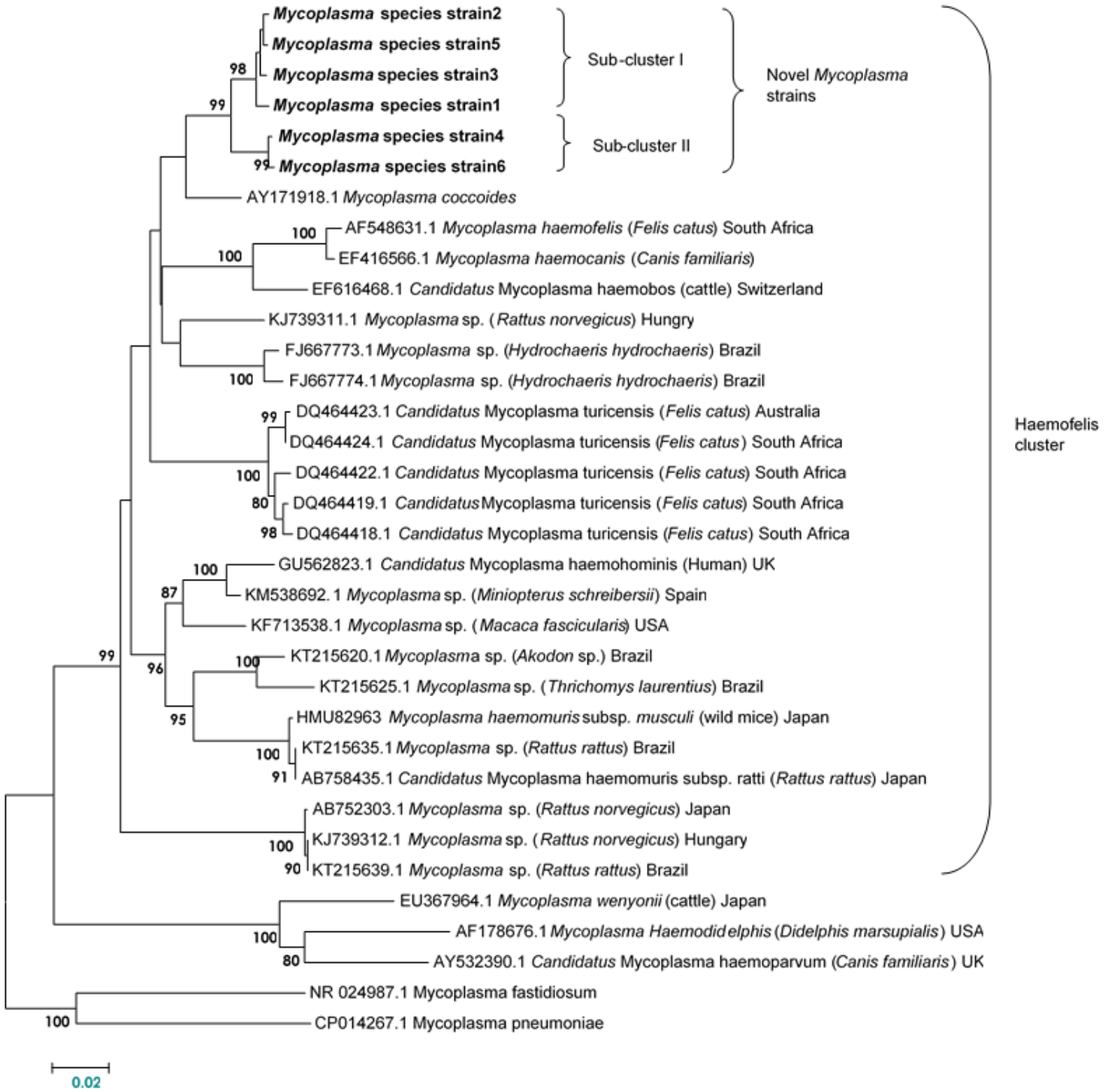

Figure 3.4: Neighbour-joining tree depicting genetic relationships of six novel haemoplasma strains detected in four bathyergid species (*Bathyergus suillus*, *Georychus capensis*, *Cryptomys hottentotus hottentotus* and *Fukomys damarensis*) sampled in the Western Cape, South Africa (indicated in bold) and relevant reference sequences. The tree was inferred using an aligned 625 nucleotide fragment of the 16S rRNA gene region. Bootstrap support values > 70% are indicated next to the relevant nodes.

3.3.5. Statistical analyses

A significant difference in overall *Mycoplasma* prevalence was found between different host species with *B. suillus* showing a higher prevalence (41.86%) compared to its close relatives (Pearson Chi-square test: $\chi^2 = 38.339$; df = 3; $p < 0.05$, Table 3.1; lung samples depicted in Figure 3.1) as well as between different sampling localities (Pearson Chi-square test: $\chi^2 = 36.745$; df = 2; $p < 0.05$), while no significant difference was found between different host sexes (Pearson Chi-square test: $\chi^2 = 0.010$; df = 1; $p = 0.921$).

In terms of strain prevalence between different hosts, significant differences between host species were found for strain 1 (Likelihood Ratio: $\chi^2 = 41.59$; df = 3; $p < 0.05$; highest in *F. damarensis*), strain 2 (Likelihood Ratio: $\chi^2 = 9.81$; df = 3; $p < 0.05$; highest in *B. suillus*), strain 4 (Pearson Chi-square test: $\chi^2 = 52.53$; df = 3; $p < 0.05$; highest in *B. suillus*) and strain 6 (Likelihood Ratio: $\chi^2 = 8.13$; df = 3; $p < 0.05$; highest in *B. suillus*), while strain 3 (Likelihood Ratio: $\chi^2 = 5.61$; df = 3; $p = 0.132$) and strain 5 (Likelihood Ratio: $\chi^2 = 2.01$; df = 3; $p = 0.570$) showed no significant difference between host species (lung samples depicted in Figure 3.2).

3.4 Discussion

In this study, we evaluated haemoplasma strain diversity and prevalence in four bathyergid species, thereby assessing haemoplasma prevalence in indigenous South African rodent populations for the first time. The high prevalence of immune-debilitating diseases, such as HIV/Aids in southern Africa, has raised public health concerns. In affected countries where communities with the highest levels of HIV infection are concentrated within informal settlements it is important to identify potential hosts and sources of emerging and re-emerging pathogens (Gratz 1999; Berg et al. 2005; Ostfeld 2009; Benacer et al. 2013; De Vries et al. 2014). Haemoplasmas represent a group of potentially fatal pathogens, for which very little data regarding their pathogenicity, diversity and distribution are available (Messick 2004; Sashida et al. 2013).

The 16S rRNA gene region has previously been used to identify and classify both haemoplasma as well as other fastidious or unculturable bacterial species (Hanage et al. 2006; Conrado et al. 2015). Furthermore, PCR approaches have been shown to be much more reliable for haemoplasma detection compared to microscopic identification from blood smears, with the latter test lacking both sensitivity and specificity compared to more robust PCR protocols (Santos

et al. 2009; Conrado et al. 2015). As the majority of genetic data available for haemoplasma strains correspond to the 16S rRNA gene (Conrado et al. 2015), developing PCR assays targeting this region, that are high in sensitivity and specificity, will aid in the rapid and accurate identification of haemoplasma strains. Given that the primer combination designed in the current study (PCR assay J, Table 3.2) showed both higher sensitivity and specificity values compared to previously published primers (PCR assay I, Table 3.2), and that PCR assay J only amplified *Mycoplasma* strains while both PCR assay I and K amplified bacterial strains belonging to other genera (Table 3.3), screening tissue and blood samples of potential haemoplasma hosts using the newly-developed primer set, will be useful for obtaining more accurate estimates of haemoplasma prevalence. It will also enhance our understanding of the distribution and diversity of haemoplasmas within mammalian species.

The observation that the haemoplasma strains detected in bathyergids grouped with haemoplasma strains belonging to the haemofelis cluster with a high level of confidence (Figure 3.3), is expected, given that both of the currently described rodent haemoplasmas, *M. coccoides* and *M. haemomuris*, fall within this cluster and that novel haemoplasma strains isolated from rodent species worldwide, also group well within this cluster (Sashida et al. 2013; Conrado et al. 2015; Gonçalves et al. 2015; Hornok et al. 2015). Other haemoplasma strains included within the haemofelis cluster include: *M. haemofelis, M. haemocanis, "Canditatus* M. turicensis", *"Candidatus* M. haemobos", as well as a human-associated haemoplasma species, "*Candidatus* M. haemohominis". Blastn results revealed that the six novel strains detected in the current study were closely related to *M. coccoides*, a haemoplasma species known to infect wild and laboratory rodents, causing acute and latent infections and resulting in haemolytic anaemia and increasing susceptibility to other diseases such as mouse hepatitis virus and lymphocytic choriomeningitis (Glasgow et al. 1971; Vieira et al. 2009).

However, as the strains identified in the current study had sequence identities of 94% - 96% to *M. coccoides*, and as it is considered that less than 97% similarity across the 16S rRNA gene is suggestive of two different species, it is probable that the strains in bathyergids represent at least one novel haemoplasma species (Drancourt & Raoult 2005). This observation is further supported by the phylogenetic analyses confirming that the newly detected haemoplasma strains formed a well-supported clade, distinct from other *Mycoplasma* species, with genetic distances between *M. coccoides* and newly detected strains exceeding those between valid haemoplasma

species (Figure 3.4). Furthermore, the observation that the novel *Mycoplasma* strains detected in the current study formed two well-supported sub-clusters, with genetic distances between these sub-clusters also exceeding the genetic distance between valid haemoplasma species, indicates that more than one novel haemoplasma species could be present within bathyergids from South Africa. Therefore, although data for alternative gene regions of haemotropic *Mycoplasma* strains are limited, characterisation of additional gene regions, such as the RNaseP (Maggi et al. 2013a), *gap*A and *dna*K gene regions (Hicks et al. 2014), for which homologous data are available, will be needed to confirm whether the strains identified in this study do indeed constitute one or more novel species.

Many novel haemoplasma strains are expected to hold zoonotic potential (Hu et al. 2004; Neimark et al. 2005; Sykes 2010; Maggi et al. 2013b; Fard et al. 2014). Therefore, the observation that *B. suillus* showed a significantly higher haemoplasma strain prevalence compared to its close relatives (Figure 3.2) and the fact that of the four strains whose prevalence was found to vary significantly between host species, three had a higher prevalence in *B. suillus* (Figure 3.3), is of relevance. This is because people living within informal settlements within the Western Cape capture and consume wild *B. suillus* populations (De Graaff et al. 1981), thereby potentially exposing themselves to bathyergid associated ectoparasites.

As bathyergids sampled in urbanised areas had a significantly higher haemoplasma prevalence and diversity compared to bathyergids sampled in a natural setting (Table 3.1; Figure 3.2; Figure 3.3), anthropogenic factors may be involved in the higher haemoplasma prevalence. Gonçalves et al. (2015) postulated that in areas disturbed by anthropogenic modification rodent species may have higher levels of haemoplasma infections compared to rodent species living in natural, pristine environments. The reason for this is that, in human transformed areas, synanthropic rodent species, such as *Rattus rattus* and *R. norvegicus*, occur. Gonçalves et al. (2015) further hypothesised these synanthropic rodent species, introduced during European colonisation may have already been infected with haemoplasma strains before their historic introduction into novel environments.

As a result, the possibility exists that in human transformed areas where synanthropic rodents co-occur with indigenous rodent species, exchange of haemoplasma pathogens through cosmopolitan arthropod vectors between these introduced species and indigenous rodent populations, is possible, particularly as *B. suillus* is a species that has been observed to occur above

ground during the day. This behaviour would, in part, explain the observed higher haemoplasma prevalence and diversity in *B. suillus*, sampled in an urban setting, compared to its more naturally occurring relatives.

Bathyergids are known to be carriers of different species of ectoparasites. Mites (*Haemolaelaps bathyergus*, *Macronyssus bacoti* and *Listroph*oriodes *bathyergians*), fleas (*Dinopsyllius ingens*), lice (*Proenderleinellus lawrensis*), and ticks (*Ixodes alluandi* and *Haemaphysalis leachii*) have been recorded on *B. suillus* (Bennett et al. 2009). Lice (*Eulinognathus hilli*) and mites (*Androlaelaps scapularis, A. capensis, A. tauffliebi* and *Radfordia* sp.) occur on *F. damarensis*, while mites (*Androlaelaps scapularis*, *A. capensis* and *Radfordia ensifera*), fleas (*Cryptopsylla ingrami*) and lice (*Eulinognathus hilli*) have been collected from *C. h. hottentotus* (Archer et al. 2014; Lutermann et al. 2015). Ectoparasites found on *G. capensis* include mites (*Haemolaelaps capensis*, *H. cryptomius*, *H. lawrencei*, and *Listrophoroides zumpti*), ticks (*Ixodes alluaudi*), and fleas (*Cryptoctenopsyllus ingens*) (Bennett et al. 2006).

As haemoplasmas are transmitted by ectoparasites, such as ticks, mites, fleas and lice, the level of ectoparasite load of the different bathyergid populations, could also impact the different levels of haemoplasma prevalence found between these populations. Although haemoplasma was not detected in the ectoparasites screened in this study, this was likely due to the very small sample size (only five fleas and two lice samples were screened). In future studies, it would be important to screen the broad range of ectoparasites that occur on mole-rats.

Ectoparasite load is expected to be influenced by both abiotic factors, such as seasonal changes in rainfall, temperature and humidity, as well as biotic factors such as changes in host behaviour, and host density within an area (Krasnov et al. 2012; Archer et al. 2014; Lutermann et al. 2015). The bathyergid populations assessed in this study occupy different biome types, with varying climatic conditions, have varying above-ground exposure and also differ in their levels of sociality and digging behaviour (Table 3.1). These factors could, at least partially, explain the differences observed in haemoplasma prevalence between host species (Gonçalves et al. 2015). Given the high haemoplasma prevalence found in bathyergid populations in the current study, further investigations of the roles ectoparasite load and diversity, as well as the roles abiotic factors, such as rainfall, play in haemoplasma transmission between bathyergid populations and, potentially, other mammals, is important.

Conclusion

The genetic data provided by this study shows, for the first time, the existence of novel haemoplasma strains in bathyergid communities. These data also highlight the potential effect of anthropogenic involvement on the spread of a blood-sucking arthropod-transmitted disease. It is important that this aspect be investigated in parallel with the role of haemoparasites in future expanded studies aimed at elucidating haemoplasma transmission dynamics. Furthermore, the PCR assay developed here demonstrates higher sensitivity and specificity relative to the two other assays assessed, and thus holds potential for improving our knowledge of haemoplasma strain distribution and diversity in wildlife populations through its application to other indigenous rodent species.

Chapter 4

General discussion

It is expected that by the year 2025 half of the world's population will live in urban areas (Gratz 1999). Most of this urban development will occur in developing countries, such as South Africa, where this accelerated expansion often exceeds the rate at which municipal services can develop adequate infrastructures to maintain healthy living conditions (Gratz 1999; Taylor et al. 2008). As a consequence, rapid urbanisation leads to significant increases in slum living conditions, and subsequently, people living in these areas need to rely more on natural bodies of water for sanitation, soil systems for small-scale agriculture, and on wildlife as alternative sources of protein (Duran-Alvarez & Jimenez-Cisneros 2014). These practices significantly alter once pristine ecosystems, through the introduction of pollutants, the destruction of biodiversity and the introduction of alien species (Berg et al. 2005; Bradley & Altizer 2007). Furthermore, these activities expose people living in poorer communities to potential reservoirs of opportunistic bacterial pathogens (Gratz 1999). Rodent species throughout the world have been implicated in the spread of various bacterial, viral, protozoan and fungal diseases (Cox 1979). However, relatively few studies have focused on potential pathogens within indigenous rodent communities in South Africa (Taylor et al. 2008). As a result, the main aim of this study was to detect and characterise potentially zoonotic bacterial pathogens in bathyergids from the Western Cape Province, an area where many people live in poor, informal settlements.

Anthropogenic activities can alter microbiota community structure and introduce opportunistic bacterial pathogens into natural environments, either intentionally through practices such as bioremediation, or unintentionally through polluting activities such as the runoff of waste water into natural soil systems (Baumgardner 2012; Duran-Alvarez & Jimenez-Cisneros 2014). These newly introduced opportunistic pathogens have the potential to propagate throughout soil systems, especially in the highly nutrient-rich rhizosphere, which can lead to the establishment of novel interactions between different classes of soil biota, such as microbe-bathyergid interactions (Berg et al. 2005; Aujoulat et al. 2012). Due to their subterranean lifestyle and reliance on the rhizosphere for food such as roots and tubers (Thomas et al. 2009), bathyergids are ideal candidate host species for investigating soil-borne pathogen reservoir and sentinel potential. In keeping with

this, the primary aim of the second chapter was to assess whether *Bathyergus suillus* and its close relatives, all belonging to the family Bathyergidae, are reservoirs of infection, or whether these mole-rat species could potentially be sentinels of anthropogenic soiling. Owing to their soil-association and potential pathogenicity (Jensen et al. 2003), members of the *Bacillus cereus* complex, represented a good candidate bacterial group for the assessment of this sentinel role. Molecular screening revealed a significantly higher prevalence of *Bacillus* bacterial strains in *B. suillus* individuals, sampled in urban environments, compared to their close relatives sampled, in natural settings. Therefore, the null hypothesis of hypothesis 1.1, which assumed that there would be no difference in prevalence between different host species and between different sampling localities, is rejected. Anthropogenic activities such as ploughing or the use of manure in agriculture, pollution of natural water and soil systems through waste water runoff or other activities such as bioremediation or biocontrol are all expected to not only alter the environment where microbe-bathyergid interactions occur, but to also have the potential to introduce opportunistic bacterial pathogens within once pristine environments (Berg et al. 2005; Jackson et al. 2009; Aujoulat et al. 2012; Duran-Alvarez & Jimenez-Cisneros 2014). As a result, these activities could have, at least partially, played a role in the observed difference in prevalence between bathyergids sampled in urban and natural settings. However, factors other than anthropogenic activities, such as specific climatic conditions, or the soil structure inhabited by bathyergid hosts could have had an effect on the observed differences in prevalence between the various mole-rat species and different sampling localities. Therefore, in future, targeted studies are needed where the specific effects of anthropogenic pollution activities, climatic conditions and soil structure on bacterial prevalence and community structure are assessed in parallel. These studies need to focus on bacterial prevalence and diversity both in surrounding environments, as well as within bathyergid and other mammalian hosts, including humans. Furthermore, as it has been shown that *Bacillus* incidence can vary seasonally in humans (Turnbull & Kramer 1985), future studies focussing on the seasonal differences of *Bacillus* incidence in bathyergids would also be of interest. These studies will lead to much needed data on below ground biodiversity and a better understanding of how the interactions between different classes of soil biota can impact human health.

A further consequence of rapid and persistent urbanisation, especially in developing countries, is that vector-borne disease incidence in humans is expected to rise substantially

(Ostfeld 2009). In natural ecosystems devoid of human influence, biodiversity is usually high with many interactions occurring between different classes of biota (Bradley & Altizer 2007). Within these environments, blood-sucking arthropods, which are potential vectors of disease, come into contact with a wide range of potential hosts, which vary in their capacity to act as reservoirs of the pathogens carried by the arthropods (Bradley & Altizer 2007). Potential hosts in these areas which are ineffective in disease transmission then create a "dilution effect" which results in a decrease in disease transmission to more effective / susceptible host species (Bradley & Altizer 2007; Ostfeld 2009). In contrast, in areas altered by humans, where species richness is low, the overall increased abundance of a few hosts, who are effective reservoir species of vector-borne diseases, can increase disease transmission (Ostfeld 2009; Kilpatrick & Randolph 2012). In developing countries, the disease burdens of vector-transmitted pathogens often remain unknown as a result of various financial and technical constraints (Kilpatrick & Randolph 2012). Haemotropic *Mycoplasma* strains represent a group of unculturable, obligate erythrocyte pathogens, which infect a wide range of mammalian hosts with some strains suspected to have zoonotic potential (Messick 2004; Hu et al. 2004; Maggi et al. 2013b). These bacterial pathogens are transmitted through blood-sucking arthropod vectors, however, data on the prevalence, diversity, distribution and exact mode of transmission of haemoplasma strains in wildlife is largely unknown (Willi et al. 2006; Gonçalves et al. 2015). Although previously found to infect humans (Tasker et al. 2010; Maggi et al. 2013c) and domestic cats (Tasker et al. 2003; Willi et al. 2006) in South Africa, and despite rodents having been identified as carriers of a range of haemoplasma strains worldwide (Hornok et al. 2015), no data on haemoplasma prevalence in indigenous rodent communities from South Africa is currently available. Consequently, the main aim of the third chapter was to assess both haemoptropic *Mycoplasma* prevalence and strain diversity in wild-caught and captive-held bathyergid species. Comparable to what was found in chapter two, molecular screening revealed that there was a significantly higher haemoplasma strain presence in *B. suillus* compared to its close relatives. As a result, the null hypothesis of hypothesis 2.1, which stated that there will be no difference in prevalence between different host species and between different sampling localities, is rejected. Instead, this chapter provided valuable data indicating that haemoplasma strains, which could be of zoonotic potential, are circulating in indigenous rodent populations within South Africa and occur at higher levels in bathyergids sampled from peri-urban localities.

In addition to screening bathyergid hosts for haemoplasma prevalence, ectoparasites found on *B. suillus* individuals were also screened, but haemoplasma strain presence was not identified in these potential vectors. However, this may have been a consequence of having a low sample size of ectoparasites (only five fleas and two lice samples were screened).

Haemoplasma prevalence in different hosts is expected to be influenced by both abiotic factors such as seasonal changes in rainfall, temperature and humidity, and biotic factors such as host mating activity and host density within an area, as all these factors can influence ectoparasite load and therefore haemoplasma transmission between hosts (Krasnov et al. 2012; Archer et al. 2014; Lutermann et al. 2015). Considering this, in future, the roles that ectoparasite load and diversity, as well as the roles that abiotic factors play in haemoplasma transmission between bathyergid populations and, potentially, other mammals, need to be assessed. Moreover, bathyergids sampled in an urban setting once again showed significantly higher haemoplasma prevalence compared to their relatives sampled in natural environments. It has been posited that indigenous rodents living in more urban environments should display higher loads of haemoplasma compared to their naturally occurring counterparts as these urban rodents come into contact with synanthropic rodents including *Rattus rattus* and *R. norvegicus*, as well as their accompanying ectoparasites, which could harbour haemoplasma strains (Gonçalves et al. 2015). As such, future studies are required to assess if the level of human transformation of areas inhabited by bathyergids, as well as other indigenous rodent species, can have an effect on haemoplasma prevalence and diversity.

The secondary aim of both chapter two and three was to assess and improve the accuracy of molecular detection and characterisation techniques for assessing the diversity and prevalence of *B. cereus* complex strains as well as haemotropic *Mycoplasma* strains present in environmental and clinical samples. The polymerase chain reaction (PCR) has repeatedly been shown to be a fast and accurate diagnostic method for determining bacterial species prevalence in both clinical and environmental samples (Gopinath & Singh 2009; McAuliffe et al. 2013). As a result, the current study assessed both the sensitivity and specificity of various available and newly-designed PCR assays to detect and characterise both *B. cereus* complex and haemoplasma strains.

In chapter two, nine different PCR assays were assessed. These targeted the 16S rRNA gene region (one assay), the *gro*EL gene region (three PCR assays), the *yea*C gene region (one PCR assay) and the *gyr*B gene region (four PCR assays). Of the gene regions assessed, the *gro*EL

gene region seemed to be the most useful for the accurate detection of *B. cereus* complex strains, with a primer set designed by Park et al. (2007) targeting this gene region detecting two strains which, according to both phylogenetic analyses and blastn searches, fell within the *B. cereus* complex with high levels of support. The data generated for this gene region allowed for the development of two additional PCR assays, also specific for the *gro*EL gene region, which could selectively amplify each of the strains in co-infected samples and which aided in determining levels of co-infection. As a result, the null hypothesis of hypothesis 1.2, which stated that PCR techniques would not be useful in detecting *B. cereus* complex strains in environmental and clinical samples, is rejected. However, for all gene regions assessed, valid *B. cereus* complex strains which served as reference sequences were interspersed throughout the phylogeny. This highlighted the difficulty in accurately characterising strains of the *B. cereus* complex to species level and confirms the notion that species belonging to the *B. cereus* group do not constitute monophyletic species (Zwick et al. 2012). Furthermore, host genome amplification occurred with PCR assays targeting both the *yea*C gene region as well as the *gyr*B gene region. This emphasises the need for future studies to identify and develop PCR assays suitable for assessing *B. cereus* complex diversity whilst avoiding non-target host genome amplification for these specific gene regions.

In chapter three, three different PCR assays (I, J and K), all targeting the 16S rRNA gene region were assessed. The first PCR assay (I) consisted of previously designed haemoplasma-specific primers (Maggi et al. 2013b), the second PCR assay (J) consisted of two newly designed primers, while the third PCR assay (K) consisted of the combination of a universal bacterial forward primer with the newly designed reverse primer, developed for PCR assay J. All three PCR assays were able to detect haemoplasma strains present in bathyergids, however, the newly designed primer set (J) had both the highest sensitivity and specificity values compared to the two other PCR assays assessed. Moreover, phylogenetic analyses revealed six novel haemoplasma strains, grouping within a discrete monophyletic lineage within the haemofelis cluster containing valid species. The null hypothesis of hypothesis 2.2, which stated that PCR techniques would not be suited to detect and characterise haemoplasma strains within bathyergid tissue, is thus rejected. Given that haemoplasma strains have not been cultivated in free cell media to date and that this has resulted in a limited understanding of both the biology and pathogenesis of these potentially zoonotic pathogens (Messick 2004; Sashida et al. 2013), the development of a rapid detection test, both high in sensitivity and specificity, as was done in the current study, will prove important for

future research into the diversity and distribution of haemoplasma strains. Due to the relatively large amount of reference data available for the 16S rRNA gene region, the current study only assessed PCR assays targeting this gene region. Whilst there are assays available that target other gene regions, one of these, the RNase P gene region amplifies a DNA fragment of only ~160bp, making this gene region of limited phylogenetic utility (Maggi et al. 2013a). The other gene regions reported in literature are limited to one study which evaluated the use of non-ribosomal gene regions, *gap*A and *dna*K, to resolve haemoplasma phylogenetic relationships (Hicks et al. 2014). Therefore, future studies are needed to assess both the sensitivity and specificity of available PCR assays targeting these alternative gene regions, and there is a need to identify additional phylogenetically informative gene regions that can aid species delineation.

With the increase in immune-debilitating diseases, such as HIV/Aids and cystic fibrosis, public health concerns have been raised, and the need to understand the disease dynamics of opportunistic pathogens have become more important (Gratz 1999; Berg et al. 2005; Ostfeld 2009; Benacer et al. 2013; De Vries et al. 2014). Developing countries in southern Africa, including South Africa, carry most of these disease burdens, without having the necessary resources to adequately prevent and treat emergent diseases (Kilpatrick & Randolph 2012). Moreover, global climate change as well as continuous anthropogenic destruction and encroachment into once pristine environments can result in the introduction of new opportunistic pathogens into natural ecosystems (Berg et al. 2005; Duran-Alvarez & Jimenez-Cisneros 2014). This, in turn, alters interactions between different classes of biota and novel microbe-mammalian interactions are established, which will most likely reflect as high levels of infections in naïve mammal hosts. However, the effect of this on human health has generally been overlooked (Berg et al. 2005; Parker 2010). Therefore, emphasis needs to be placed on identifying both potential hosts of emerging and re-emerging pathogens as well as identifying the environmental and anthropogenic conditions that facilitate the transfer of these pathogens from the environment to potential animal reservoirs, between different animal reservoirs, as well as between animal reservoirs and humans. The establishment of continuous surveillance programmes which incorporate fast and accurate detection methods, such as those evaluated in the current study, will not only aid in mitigating the risk of humans contracting opportunistic pathogens from their surroundings, but also aid in gaining a vital understanding of how human activities are impacting and changing natural systems, all of

which serves to emphasise why keeping natural systems intact is important for human health and subsistence.

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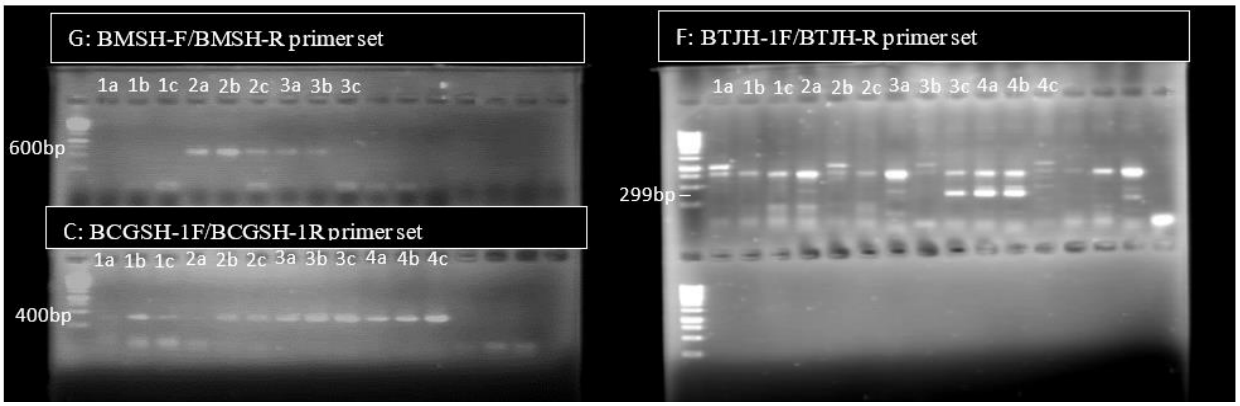
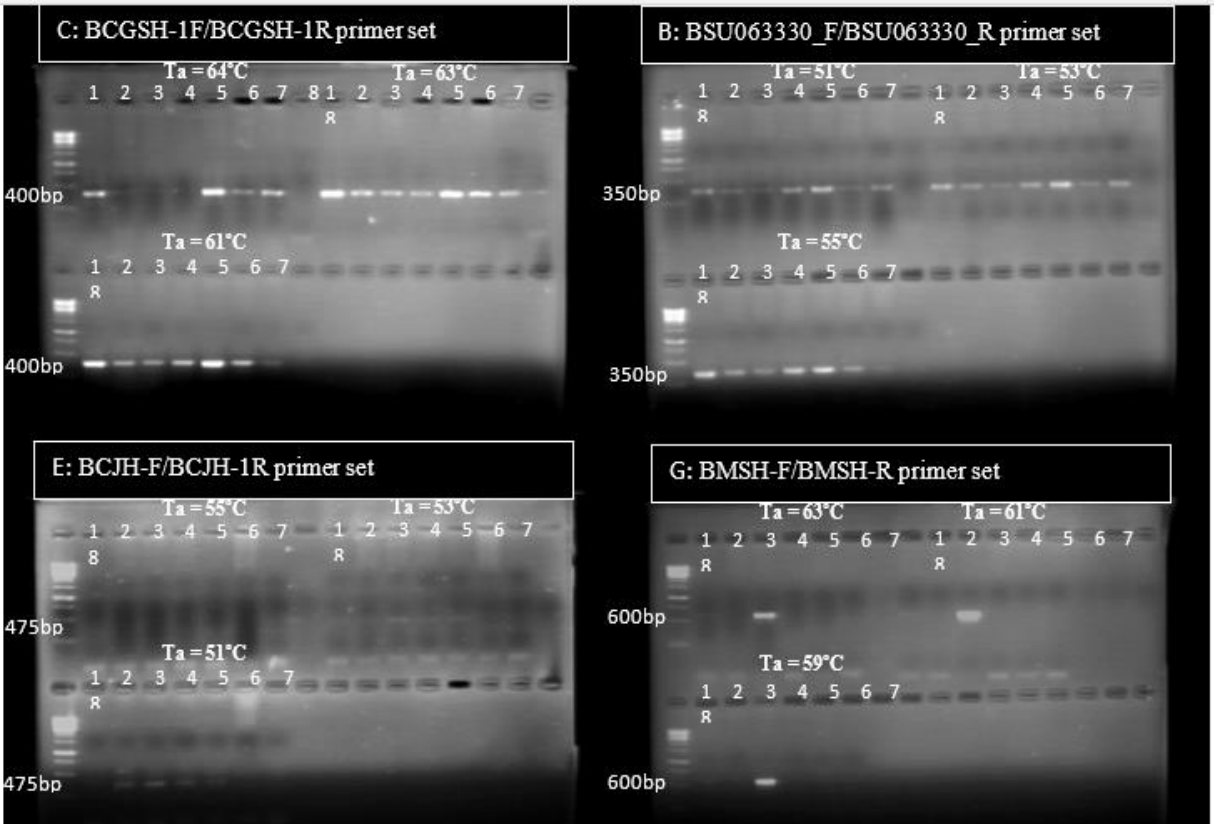
6. Appendix



Appendix 2.1.: Results of primer ranging of primer sets BMSH-F/BMSH-R (G), BCGSH-1F/BCGSH-1R (F) and BTJH-1F/BTJH-R (C); numbers (1 – 4) indicate samples run at different primer concentrations (a – c) with different primer sets (G, F and C). Primer concentrations are a = 0.25 µM, b = 0.5 µM, c = 0.75 µM d = 1 µM respectively for each sample run. Expected amplicon size is indicated for each primer set next to the 1kb molecular marker (Fermentas, Waltham, Massachusetts, USA). Agarose gel of 2.5% used.



Appendix 2.2: Results of temperature ranging of primer sets BCGSH-1F/BCGSH-1R (C), BSU063330_F/BSU063330_R (B), BCJH-1F/BCJH-R (E) and BMSH-F/BMSH-R (G). Individual samples are indicated by numbers 1 – 7 and 8 represents the negative control. Final annealing temperatures and expected size of amplicon are indicated for each primer set. Expected amplicon size is indicated for each primer set next to the 1kb molecular marker (Fermentas, Waltham, Massachusetts, USA). Agarose gel of 2.5% used.

Appendix 3.1: Alignment created in Mega 6 (Tamura et al. 2013). Dots (.) indicate base pair similarities while dashes (-) indicate insertions or deletions.

```
AB758434.1_Candidatus_Mycoplasma_haemomuris              GGCGAACGGG TGAGTAATGA ATACTTAACA TACCTCCATG AAGGAAATAG CTATTCGAAA GAGTAATTAA TGTCCTATAG GAGCCAGCCC CACA-TGAGG TTGGCTTTAA AGGCGCAAGC
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... ....-..... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .....TT..T ....-..... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .........T ....-..... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .....TT..T ....-..... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            .........C .........G .G..C.GGA. .G..G..... .CGCC..... .G.CG..... .CC..C.... .....CCGA. ..AGAA.GAC GG.A..... ...A....T.
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .....TT..T ....-..... .......... ..........
AY171918.1_Mycoplasma_coccoides                          .......... ........AC ...T...... ....C.TTA. .G........ .CG.CT.... AGACG..... .....C.... ..A..CC.-T ....-G.... GGTTT..... ...A....T.
DQ157156.1_Mycoplasma_haemofelis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .CGC.T.... A..CG..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ464417.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ464418.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ464419.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ464420.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ464422.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CTACG. .......... .CG....... ..ACG..... .......... T.T..TC..T ..G.CA..A. GG..A..... ...T.A..A.
DQ464423.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..C..CG. .......... .CG....... ..ACG..... .......... T.T..CCTTT ..G.CA..AA GGA.A..... ...T....A.
DQ464424.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..C..CG. .......... .CG....... ..ACG..... .......... T.T..CCTTT ..G.CA..AA GGA.A..... ...T....A.
DQ825448.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ825454.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CTTCG. .......... .CG....... ..A.G..... .......... T.T.TTT... ..A.-A.GA. GAA.A..... ...T.A..A.
EF416566.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
EF416567.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
AF178677.1_Haemobartonella_felis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .CGC.T.... A..CG..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
EU839977.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
EF416568.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
GQ129115.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTC..G
GQ129116.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
GQ129117.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
GQ129118.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AGTTTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
GQ129119.1_Mycoplasma_haemocanis                         ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
GU562823.1_Candidatus_Mycoplasma_haemohominis            .......... ........AC ...T...... ....C..CAA ...TGC.... ...CC..... .G........ .ACA..... ...G.TC.GA ....-A.TC. GG.T..... ....CTTG..
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia     ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..T.TTCG.A
NR_074289.1_Mycoplasma_haemocanis                        ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .C.C.T.... A...G..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C.TTCG.G
NR_103953.1_Mycoplasma_haemofelis                        ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .CGC.T.... A..CG..... .AC..C.... ..AG.TTTAT .TAT--..TT .A.CT..... ..C.TTCG.G
U88563.2_Mycoplasma_haemofelis                           ...A...... ........AC ...TC..... .G..C.TC.. TG..GG.... .CGC.T.... A..CG..... .AC..C.... ..AG.TTTAT .CAT--..TT .A.CT..... ..C-TTCG.G
DQ157150.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ157152.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ157153.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.
DQ157154.1_Candidatus_Mycoplasma_turicensis               .......... ........AC ...T...... .G..CT.CG. .......... .CG....... ..ACG..... .......... T.T..TC.AT ..G.CA..A. GG..A..... ...T.A..A.


AB758434.1_Candidatus_Mycoplasma_haemomuris              CACTTGGAGA TTGGAGTATT TTCTATTAGC TAGTTGGCGG GATAATAGCC CACCAAGGCA GTGATAGATA GCTGGTCTAA GAGGATGAAC AGCCACAATG GGATTGAGAT ACGGCCCATA
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            .G.CCC.G.. .........G .......... .T........ .G...A.... .........G A......... .......... .......... .......... .......... ..........
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY171918.1_Mycoplasma_coccoides                          .G...T.G.. .....A...G C........T .......... .G...AG... .......A.T A......... ........T. .....C.... .......... .......... ..........
DQ157156.1_Mycoplasma_haemofelis                         .G..GA.G.. ....GA...G C......... .......... .....A.... .......... A......... ........T. .......... .......... .......... ..........
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ464417.1_Candidatus_Mycoplasma_turicensis               .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ464418.1_Candidatus_Mycoplasma_turicensis               .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ464419.1_Candidatus_Mycoplasma_turicensis               .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ464420.1_Candidatus_Mycoplasma_turicensis               .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ464422.1_Candidatus_Mycoplasma_turicensis               .G.CGAAG.. .....A...G .C........ .......... .....A.... .......... A......G.. .......... .......... .......C. .......... ..........
DQ464423.1_Candidatus_Mycoplasma_turicensis               .G.CGA.G.. .....A...G .C........ .......... .....A.... .......... A......G.. .......... .......... .......... .......... ..........
DQ464424.1_Candidatus_Mycoplasma_turicensis               .G.CGA.G.. .....A...G .C........ .......... .....A.... .......... A......G.. .......... .......... .......... .......... ..........
DQ825448.1_Candidatus_Mycoplasma_turicensis               .G.CG.AG.. .....A...G .C........ .......... .....A.... .........G A......G.. .......... .......... .......... .......... ..........
DQ825454.1_Candidatus_Mycoplasma_turicensis               .G.CGAAG.. .....A...G .C........ .......... .G...A.... .........G A......G.. .......... .......... .......... .......... ..........
EF416566.1_Mycoplasma_haemocanis                         .G..GA.G.. ....GA...G C......... .......... .....A.... .......... A......... ........T. .......... .......... .......... ..........
EF416567.1_Mycoplasma_haemocanis                         .G..GA.G.. ....GA...G C......... .......... .....A.... .......... A......... ........T. .......... .......... .......... ..........
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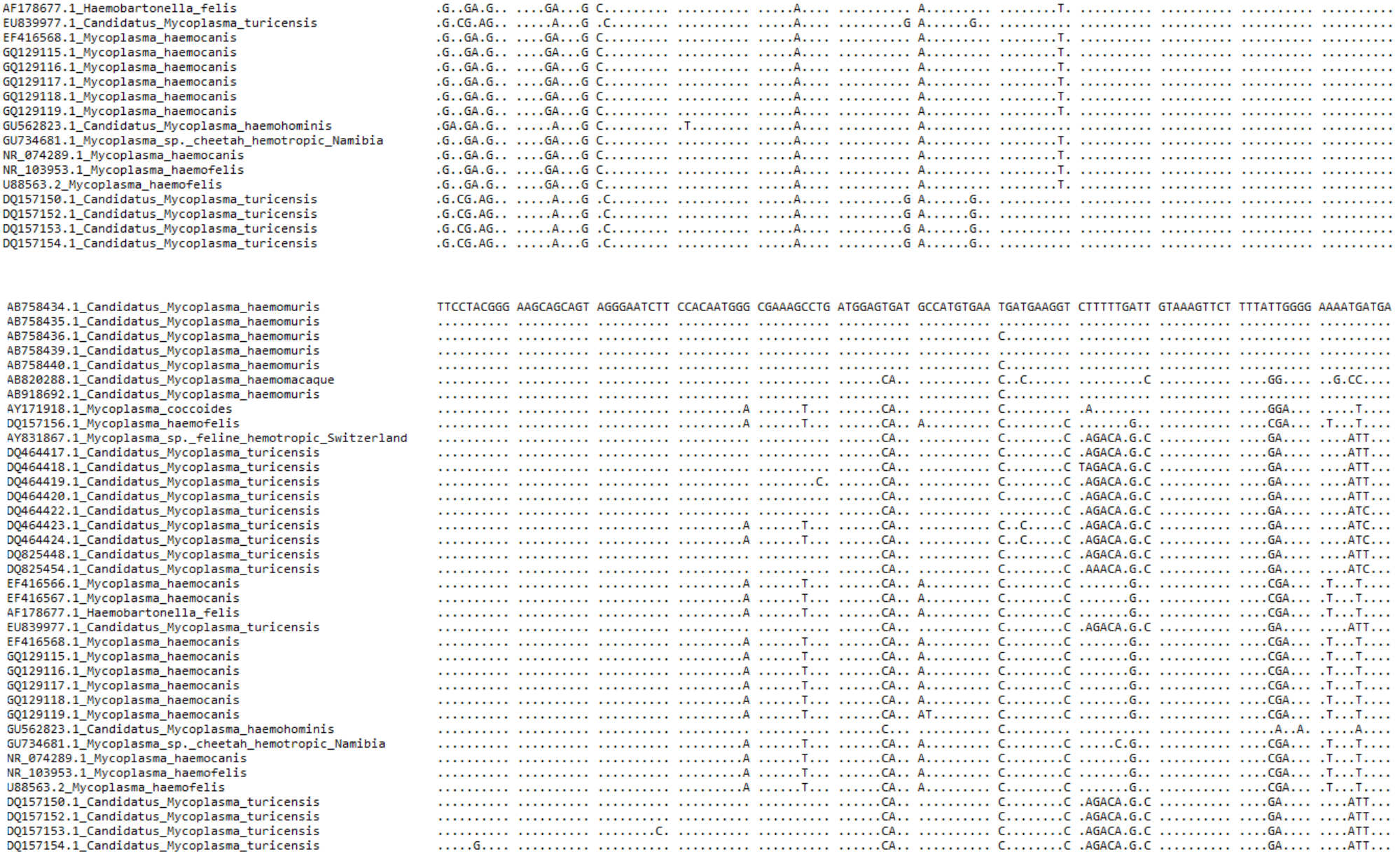
AF178677.1_Haemobartonella_felis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
EU839977.1_Candidatus_Mycoplasma_turicensis .G.CG.AG.. .....A...G .C..... ....A.... .....G A.....G.. ....
EF416568.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GQ129115.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GQ129116.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GQ129117.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GQ129118.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GQ129119.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
GU562823.1_Candidatus_Mycoplasma_haemohominis .GA.GA.G.. .....A...G C..... .T..... .....A.... ..... A..... ....
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
NR_074289.1_Mycoplasma_haemocanis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
NR_103953.1_Mycoplasma_haemofelis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
U88563.2_Mycoplasma_haemofelis .G..GA.G.. ....GA...G C..... ....A.... ..... A..... ....T. ....
DQ157150.1_Candidatus_Mycoplasma_turicensis .G.CG.AG.. .....A...G .C..... ....A.... ..... G A.....G.. ....
DQ157152.1_Candidatus_Mycoplasma_turicensis .G.CG.AG.. .....A...G .C..... ....A.... ..... G A.....G.. ....
DQ157153.1_Candidatus_Mycoplasma_turicensis .G.CG.AG.. .....A...G .C..... ....A.... ..... G A.....G.. ....
DQ157154.1_Candidatus_Mycoplasma_turicensis .G.CG.AG.. .....A...G .C..... ....A.... ..... G A.....G.. ....
AB758434.1_Candidatus_Mycoplasma_haemomuris TTCCTACGGG AAGCAGCAGT AGGGAATCTT CCACAATGGG CGAAAGCCTG ATGGAGTGAT GCCATGTGAA TGATGAAGGT CTTTTTGATT GTAAAGTTCT TTTATTGGGG AAAATGATGA
AB758435.1_Candidatus_Mycoplasma_haemomuris ....
AB758436.1_Candidatus_Mycoplasma_haemomuris .... C..... ....
AB758439.1_Candidatus_Mycoplasma_haemomuris ....
AB758440.1_Candidatus_Mycoplasma_haemomuris .... C..... ....
AB820288.1_Candidatus_Mycoplasma_haemomacaque ....CA.. ..... C..C..... ....C ....GG.... ..G.CC....
AB918692.1_Candidatus_Mycoplasma_haemomuris .... C..... ....
AY171918.1_Mycoplasma_coccoides ....A .....T... ......CA.. ..... C..... .A..... ....GGA... .....T....
DQ157156.1_Mycoplasma_haemofelis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ464417.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ464418.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C TAGACA.G.C ....GA.... ....ATT...
DQ464419.1_Candidatus_Mycoplasma_turicensis ....C. .....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ464420.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ464422.1_Candidatus_Mycoplasma_turicensis ....CA.. ....C .AGACA.G.C ....GA.... ....ATC...
DQ464423.1_Candidatus_Mycoplasma_turicensis ....A .....T... ......CA.. ..... C..C.....C .AGACA.G.C ....GA.... ....ATC...
DQ464424.1_Candidatus_Mycoplasma_turicensis ....A .....T... ......CA.. ..... C..C.....C .AGACA.G.C ....GA.... ....ATC...
DQ825448.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ825454.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AAACA.G.C ....GA.... ....ATC...
EF416566.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
EF416567.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
AF178677.1_Haemobartonella_felis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
EU839977.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
EF416568.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
GQ129115.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
GQ129116.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
GQ129117.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
GQ129118.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
GQ129119.1_Mycoplasma_haemocanis ....A .....T... ......CA.. AT..... C.....C .....G.. ....CGA... .T...T....
GU562823.1_Candidatus_Mycoplasma_haemohominis ....C... ..... C..... ....A..A. .....A....
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia ....A .....T... ......CA.. A..... C.....C .....C.G.. ....CGA... .T...T....
NR_074289.1_Mycoplasma_haemocanis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
NR_103953.1_Mycoplasma_haemofelis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
U88563.2_Mycoplasma_haemofelis ....A .....T... ......CA.. A..... C.....C .....G.. ....CGA... .T...T....
DQ157150.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ157152.1_Candidatus_Mycoplasma_turicensis ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ157153.1_Candidatus_Mycoplasma_turicensis ....C. ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...
DQ157154.1_Candidatus_Mycoplasma_turicensis .....G.... ....CA.. ..... C.....C .AGACA.G.C ....GA.... ....ATT...

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AB758434.1_Candidatus_Mycoplasma_haemomuris              TGGTACCCAG TGAATAAGTG ACAGCAAACT ATGTGCCAGC AGCTGCGGTA ATACATAGGT CGCGAGCGTT ATTCGGATTT ATTGGGCGTA AAGCGAGCGC AGGCGGATTG GTAAGTTCTG
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            C.......CT .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A........A
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY171918.1_Mycoplasma_coccoides                          .......TCC .......... .......... .......... .......... .......... ...A...... .......... .......... ....A..... ........GA AC........
DQ157156.1_Mycoplasma_haemofelis                         .A....TTC. .......... .......... .......... .......... .......... .......A.. .......... .......... ....A..... ........GT ..........
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464417.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464418.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ...T....GA A.........
DQ464419.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464420.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464422.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464423.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ464424.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ825448.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ825454.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
EF416566.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
EF416567.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
AF178677.1_Haemobartonella_felis                         .A....TTC. .......... .......... .......... .......... .......... .......A.. .......... .......... ....A..... ........GT ..........
EU839977.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
EF416568.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GQ129115.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GQ129116.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GQ129117.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GQ129118.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GQ129119.1_Mycoplasma_haemocanis                         .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
GU562823.1_Candidatus_Mycoplasma_haemohominis            .T...T..TA .......... .......... .......... .......... .......... .......... .......... .......... ....A..... .......CG. AC.....T..
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia     .A....TTC. .......... .......... .......... .......... .......... .......A.. .......... .......... ....A..... ........GT ..........
NR_074289.1_Mycoplasma_haemocanis                        .A....TTC. .......... .......... .......... .......... .......... ...A...A.. .......... .......... ....A..... ........GT ..........
NR_103953.1_Mycoplasma_haemofelis                        .A....TTC. .......... .......... .......... .......... .......... .......A.. .......... .......... ....A..... ........GT ..........
U88563.2_Mycoplasma_haemofelis                           .A....TTC. .......... .......... .......... .......... .......... .......A.. .......... .......... ....A..... ........GT ..........
DQ157150.1_Candidatus_Mycoplasma_turicensis              ........TC .......... ........T. .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ157152.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ157153.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......... .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
DQ157154.1_Candidatus_Mycoplasma_turicensis              ........TC .......... .......G.. .......... .......... .......... .......... .......... .......... ....A..... ........GA A.........
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AB758434.1_Candidatus_Mycoplasma_haemomuris              TGTTAAATGC AGCCGCTCAA CGGTTGTATG CGCAGAATAC TGCTTTTCTA GAATACGGTA GAAAGTTTTG GAATTGAATG TGGAGCGGTG GAATGTGTAG ATATATTCAA GAACACCAGA
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... ...C...... .......... .......... .......... .......... .......... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... ...C...... .......... .......... .......... .......... .......... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            ........A. ...T...... .A.....T.. ..TC...... ..T.CA.... ....GT.... .GCG...... .........A .......... .......... .......... ..........
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... ...C...... .......... .......... .......... .......... .......... .......... ..........
AY171918.1_Mycoplasma_coccoides                          .......A.. ...T...... .A.....T.. .A.C...... ..T.CG.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ157156.1_Mycoplasma_haemofelis                         .......... ...TA..... TA........ .A.C...... .A.A.G.... ..T.GT.... .GG.....C. .....A.GCA .......... .......... ....GC.T.. ..........
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464417.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464418.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464419.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464420.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464422.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ464423.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .......... .....A...A .......... ........G. .......T.. ..........
DQ464424.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .......... .....A...A .......... .......... .......T.. ..........
DQ825448.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .G........ .....A...A .......... .......... .......T.. ..........
DQ825454.1_Candidatus_Mycoplasma_turicensis              CA.....A.. ...T...T.. .A.....T.. T..C...... .AT.CA.... ....GT.... .......... .....A...A .......... .......... .......T.. ..........
EF416566.1_Mycoplasma_haemocanis                         .......... ...TA..... TA........ .A.C...... .A.A.G.... ..T.GT.... .GG.....C. .....A.GCA .......... .......... ....GC.T.. ..........
EF416567.1_Mycoplasma_haemocanis                         .......... ...TA..... TA........ .A.C...... .A.A.G.... ..T.GT.... .GG.....C. .....A.GCA .......... .......... ....GC.T.. ..........
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AF178677.1_Haemobartonella_felis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
EU839977.1_Candidatus_Mycoplasma_turicensis       CA.....A..  ...T...T..  .A.....T..  T..C......  .AT.CA....  ....GT....  .G........  .....A...A  ..........  ..........  .......T..  ..........
EF416568.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GQ129115.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GQ129116.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GQ129117.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GQ129118.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GQ129119.1_Mycoplasma_haemocanis                  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
GU562823.1_Candidatus_Mycoplasma_haemohominis      .......A..  ..TT......  .AA....T..  ...T......  ..TCCG....  ..........  .G........  .....T...A  ..........  ..........  .......A..  ..........
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia  ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
NR_074289.1_Mycoplasma_haemocanis                 ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
NR_103953.1_Mycoplasma_haemofelis                 ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
U88563.2_Mycoplasma_haemofelis                    ..........  ...TA.....  TA........  .A.C......  .A.A.G....  ..T.GT....  .GG.....C.  .....A.GCA  ..........  ..........  ....GC.T..  ..........
DQ157150.1_Candidatus_Mycoplasma_turicensis       CA.....A..  ...T...T..  .A.....T..  T..C......  .AT.CA....  ....GT....  .G........  .....A...A  ..........  ..........  .......T..  ..........
DQ157152.1_Candidatus_Mycoplasma_turicensis       CA.....A..  ...T...T..  .A.....T..  T..C......  .AT.CA....  ....GT....  .G........  .....A...A  ..........  ..........  .......T..  ..........
DQ157153.1_Candidatus_Mycoplasma_turicensis       CA.....A..  ...T...T..  .A.....T..  T..C......  .AT.CA....  ....GT....  .G........  .....A...A  ..........  ..........  .......T..  ..........
DQ157154.1_Candidatus_Mycoplasma_turicensis       CA.....A..  ...T...T..  .A.....T..  T..C......  .AT.CA....  ....GT....  .G........  .....A...A  ..........  ..........  .......T..  ..........
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AB758434.1_Candidatus_Mycoplasma_haemomuris       GGCGAAGGCG  AAAACTTAGG  CCGATATTGA  CGCTTAGGCT  CGAAAGTGTG  GGGAGCAAAT  GGGATTAGAT  ACCCCAGTAG  TCCACACCGT  AAACGATGGA  TATTAG-ATG  TTGGGACTTG
AB758435.1_Candidatus_Mycoplasma_haemomuris       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque     ..........  .....CG...  ..AC......  ..........  T.........  ..........  ..........  ..........  ..........  ..........  ......-...  .....GTC..
AB918692.1_Candidatus_Mycoplasma_haemomuris       ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
AY171918.1_Mycoplasma_coccoides                   ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-G..  CC...GT.A.
DQ157156.1_Mycoplasma_haemofelis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ464417.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......G...  .C....T...
DQ464418.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ464419.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ464420.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ464422.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ464423.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C........
DQ464424.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C........
DQ825448.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ825454.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
EF416566.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
EF416567.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
AF178677.1_Haemobartonella_felis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
EU839977.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
EF416568.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GQ129115.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GQ129116.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GQ129117.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GQ129118.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GQ129119.1_Mycoplasma_haemocanis                  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
GU562823.1_Candidatus_Mycoplasma_haemohominis     ..........  ..........  ...T......  ..........  T.........  ..........  ..........  ..........  ..........  ..........  ......-...  ..........
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia  ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
NR_074289.1_Mycoplasma_haemocanis                 ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
NR_103953.1_Mycoplasma_haemofelis                 ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  ..........  .........G  ......-..A  ..A..G...T
U88563.2_Mycoplasma_haemofelis                    ..........  G.........  ..ATA.A...  ..........  T.........  ..........  ..........  ..........  .....G....  .........G  ......-..A  ..A..G...T
DQ157150.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ157152.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
DQ157153.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ...G......  .........G  ......-...  .C....T...
DQ157154.1_Candidatus_Mycoplasma_turicensis       ..........  ..........  ..AT......  ..........  T.........  ..T.......  ..........  ..........  ..........  .........G  ......-...  .C....T...
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AB758434.1_Candidatus_Mycoplasma_haemomuris              AGTCTCAGCG TTGTAGCTTA CGTTGTTAAA TATCCCGCCT GAGTAGTACA TATGCAAATA TGAAACTCAA AGGAATTGAC GGGGACCTGA ACAAGTGGTG GAACATGTTG CTTAATTCGA
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... ...G...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... ...A...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            T.C....... .......... ...G...... .......... .G........ .......... .......... .......... .......... .......... .......... ..........
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... ...C...... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY171918.1_Mycoplasma_coccoides                          ..CT..G.T. C......... .......... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ157156.1_Mycoplasma_haemofelis                         ..CT.T..T. .......... ..CC...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  T..T..G... .......... ...G...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464417.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...G...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464418.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... .......... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464419.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...A...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464420.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...A...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464422.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... .......... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464423.1_Candidatus_Mycoplasma_turicensis              ...T..G... .......... ...A...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ464424.1_Candidatus_Mycoplasma_turicensis              ...T..G... .......... ...C...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ825448.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...G-..... ..C....... .G........ .......... .........T .......... .......... .......... .......... ..........
DQ825454.1_Candidatus_Mycoplasma_turicensis              .A.T..G... .......... ...-...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
EF416566.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
EF416567.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
AF178677.1_Haemobartonella_felis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
EU839977.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...-...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
EF416568.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GQ129115.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GQ129116.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GQ129117.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GQ129118.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GQ129119.1_Mycoplasma_haemocanis                         ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
GU562823.1_Candidatus_Mycoplasma_haemohominis            C......... .......... ...-...... .......... .G........ .......... .......... .......... .......... .......... .......... ..........
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia     ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
NR_074289.1_Mycoplasma_haemocanis                        ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
NR_103953.1_Mycoplasma_haemofelis                        ..CT.T..T. .......... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
U88563.2_Mycoplasma_haemofelis                           ..CT.T..T. ...AT..... ..C-...... ..C....... .G........ .......... .......... .......... .......... .......... ..G....... ..........
DQ157150.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...-...... ..C....... .G........ .......... .......... ....G..... .......... .......... .......... ..........
DQ157152.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...-...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ157153.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...-...... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........
DQ157154.1_Candidatus_Mycoplasma_turicensis              T..T..G... .......... ...G-..... ..C....... .G........ .......... .......... .......... .......... .......... .......... ..........


AB758434.1_Candidatus_Mycoplasma_haemomuris              CAATACACGA AAAACCTTAC CAAGATTTGA CATCCCCTGC AAAGCTTTAG AAATAAAG-T GGAGGTTATC AGGGTGACAG GTGGTGCATG GCTGTCGTCA GCTCGTGTCA TGAGATGTCT
AB758435.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... ........-. .......... .......... .......... .......... .......... ..........
AB758436.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... G......... ........-. .......... .......... .......... .......... .......... ..........
AB758439.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... ........-. .......... .......... .......... .......... .......... ..........
AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... G......... ........-. .......... .......... .......... .......... .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            T......... .......... .......... ....T..C.. G....CA... .....TG.TC AT........ G..A...... .......... .T........ .......... ........T.
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... G......... ........-. .......... .......... .......... .......... .......... ..........
AY171918.1_Mycoplasma_coccoides                          T......... .G........ ....G..... ......TC.. ...A.CA... .....TG.-C .......... GA........ .......... .T........ .......... ........T.
DQ157156.1_Mycoplasma_haemofelis                         T......... .......... ....G..... ......TC.. ......A... .....T..-. A......... GA........ .......... .T........ .........T ........T.
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland  T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464417.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464418.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464419.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464420.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464422.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ464423.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... .....T.... ......A... .....T..-. .......... ..A....... .......... .T........ .......... ........T.
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DQ825448.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....GC.... .....TT... .....CA... .....TG.-. .......... ..A....... .......... .T........ .......... ........T.
DQ825454.1_Candidatus_Mycoplasma_turicensis              T......... .......... ....G..... ....TTT... G.....A... .....T..-. .......... .AAA...... .....A.... .T........ .......... ........T.
EF416566.1_Mycoplasma_haemocanis                         T......... .......... ....G..... ......TC.. ......A... .....T..-. A......... GA........ .......... .T........ .......... ........T.
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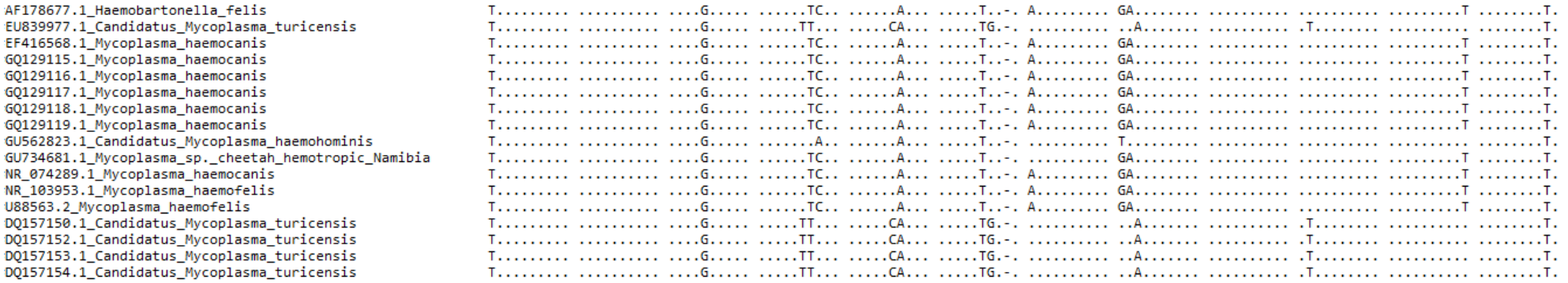
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GQ129115.1_Mycoplasma_haemocanis
GQ129116.1_Mycoplasma_haemocanis
GQ129117.1_Mycoplasma_haemocanis
GQ129118.1_Mycoplasma_haemocanis
GQ129119.1_Mycoplasma_haemocanis
GU562823.1_Candidatus_Mycoplasma_haemohominis
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia
NR_074289.1_Mycoplasma_haemocanis
NR_103953.1_Mycoplasma_haemofelis
U88563.2_Mycoplasma_haemofelis
DQ157150.1_Candidatus_Mycoplasma_turicensis
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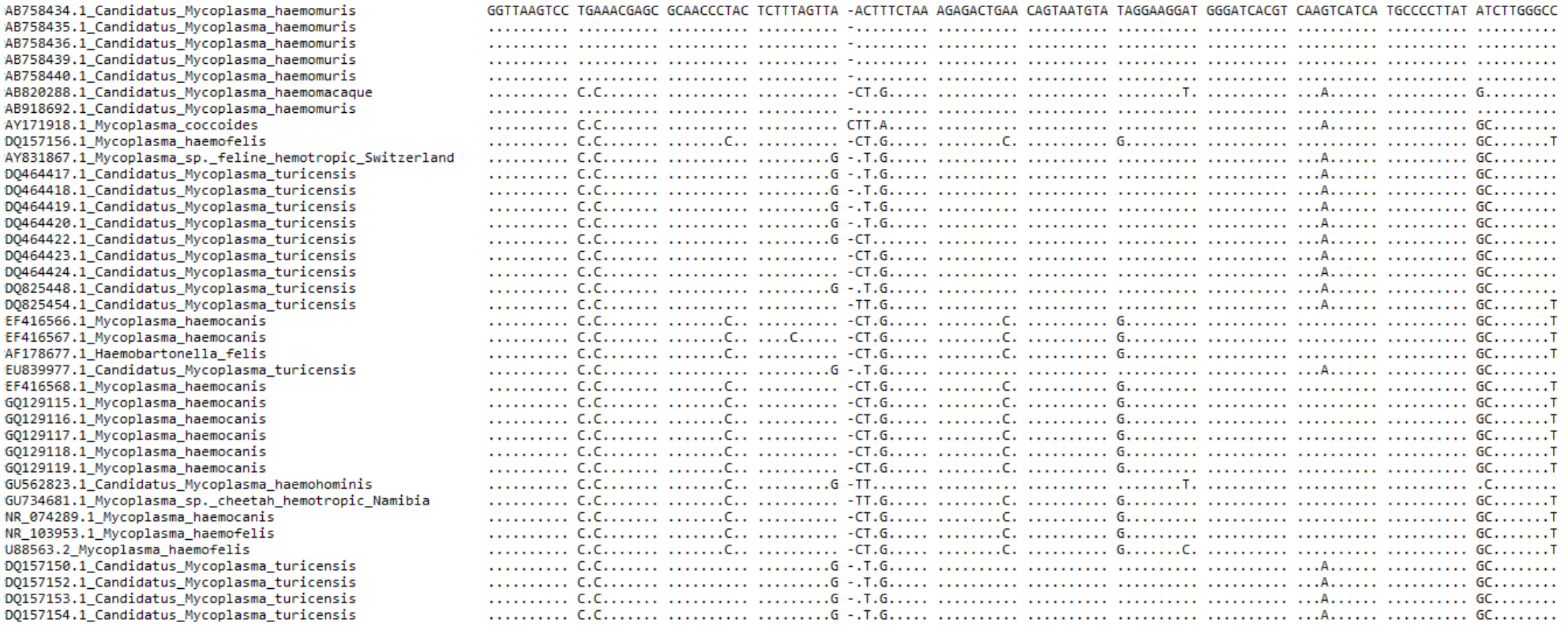
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AB758435.1_Candidatus_Mycoplasma_haemomuris
AB758436.1_Candidatus_Mycoplasma_haemomuris
AB758439.1_Candidatus_Mycoplasma_haemomuris
AB758440.1_Candidatus_Mycoplasma_haemomuris
AB820288.1_Candidatus_Mycoplasma_haemomacaque
AB918692.1_Candidatus_Mycoplasma_haemomuris
AY171918.1_Mycoplasma_coccoides
DQ157156.1_Mycoplasma_haemofelis
AY831867.1_Mycoplasma_sp._feline_hemotropic_Switzerland
DQ464417.1_Candidatus_Mycoplasma_turicensis
DQ464418.1_Candidatus_Mycoplasma_turicensis
DQ464419.1_Candidatus_Mycoplasma_turicensis
DQ464420.1_Candidatus_Mycoplasma_turicensis
DQ464422.1_Candidatus_Mycoplasma_turicensis
DQ464423.1_Candidatus_Mycoplasma_turicensis
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EF416567.1_Mycoplasma_haemocanis
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EF416568.1_Mycoplasma_haemocanis
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GQ129119.1_Mycoplasma_haemocanis
GU562823.1_Candidatus_Mycoplasma_haemohominis
GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia
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NR_103953.1_Mycoplasma_haemofelis
U88563.2_Mycoplasma_haemofelis
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DQ157152.1_Candidatus_Mycoplasma_turicensis
DQ157153.1_Candidatus_Mycoplasma_turicensis
DQ157154.1_Candidatus_Mycoplasma_turicensis

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AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .......... .......... .......... .......... ...C...... .......C.. ..C....... .......... ........-. .......... ..........
AB820288.1_Candidatus_Mycoplasma_haemomacaque            .......... .......... A......T.. ..T....... G....C..T. .......... .......... .......... .......... ........-. .......... ..........
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AY171918.1_Mycoplasma_coccoides                          .......... ........GA .......T.. .......A.T ......A.T. .......... ....-..CTC ..C....T.. .......... ........-. .......... ..........
DQ157156.1_Mycoplasma_haemofelis                         .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
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DQ464417.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........A. .......... ..........
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DQ464420.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
DQ464422.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .......C.. .......... .......... ....-..... .......... .......... ........-. .......... ..........
DQ464423.1_Candidatus_Mycoplasma_turicensis              .......... ........C. A......T.. ..T....A.. ........T. ...C...... C...-A...G .......... .......... ........-. .......... ..........
DQ464424.1_Candidatus_Mycoplasma_turicensis              .......... ........C. A......T.. ..T....A.. ........T. ...C...... C...-A...G .......... .......... ........-. .......... ..........
DQ825448.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
DQ825454.1_Candidatus_Mycoplasma_turicensis              .......... ........C. A......T.. .......AT. ........T. .......... ....-A...G .......... .......... ........-. .......... ..........
EF416566.1_Mycoplasma_haemocanis                         .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
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AF178677.1_Haemobartonella_felis                         .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
EU839977.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
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GQ129115.1_Mycoplasma_haemocanis                         .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
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GQ129118.1_Mycoplasma_haemocanis                         .........C ........C. AAC....T.. ..T....AT. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
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GU734681.1_Mycoplasma_sp._cheetah_hemotropic_Namibia     .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
NR_074289.1_Mycoplasma_haemocanis                        .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
NR_103953.1_Mycoplasma_haemofelis                        .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
U88563.2_Mycoplasma_haemofelis                           .........C ........C. AAC....T.. ..T....A.. ........T. .......... C...-TT..G .......T.. ....GG.... ........-. ...CC..... ..........
DQ157150.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
DQ157152.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......??. .......... ........-. .......... ..........
DQ157153.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
DQ157154.1_Candidatus_Mycoplasma_turicensis              .......... .......... A......T.. .....G.A.. ........T. .......... C...-A.... .......... .......... ........-. .......... ..........
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AB758440.1_Candidatus_Mycoplasma_haemomuris              .......... .........C .......... .......... .......... .......... .......... ...
AB820288.1_Candidatus_Mycoplasma_haemomacaque            .......... .......... .......... .......... .......... .......... .......... ...
AB918692.1_Candidatus_Mycoplasma_haemomuris              .......... .........C .......... .......... .......... .......... .......... ...
AY171918.1_Mycoplasma_coccoides                          .......... .......... .......... .......... .......... .......... .......... ...
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EF416567.1_Mycoplasma_haemocanis                         .......... .......... .......... ....C..... .......... .......... .......... ...
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AF178677.1_Haemobartonella_felis                    .......... .......... .......... ....C..... .......... .......... .......... ...
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NR_074289.1_Mycoplasma_haemocanis                   .......... .......... .......... ....C..... .......... .......... .......... ...
NR_103953.1_Mycoplasma_haemofelis                   .......... .......... .......... ....C..... .......... .......... .......... ...
U88563.2_Mycoplasma_haemofelis                      .......... .......... .......... ....C..... .......... .......... .......... ...
DQ157150.1_Candidatus_Mycoplasma_turicensis          .......... .......... .......... .......... .......... .......... .......... ...
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