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Molecular diversity and prevalence of *Helicobacter*, *Bartonella* and *Streptococcus* in *Mus musculus* from sub-Antarctic Marion Island in relation to host diversity

By

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

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Declaration

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

Signature:

Date **:** 9/12/2011

Disclaimer

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

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General abstract

The house mouse (*Mus musculus*) was introduced to Marion Island by sealers in the early 1800's and was found abundantly over the entire Island as early as 1818. Despite the abundance of this rodent, little information is known about the invasive house mouse from Marion Island with regards to its genetics and infectious disease potential. Therefore, this study aimed to determine the genetic diversity of the house mouse *Mus musculus* on Marion Island, as well as the prevalence of three zoonotic bacterial disease agents viz. *Bartonella*, *Helicobacter* and *Streptococcus*.

To determine the genetic diversity of *Mus musculus* from Marion Island a sub population of 91 mice was used for mitochondrial DNA sequence analysis. Sequencing the mitochondrial control region resulted in 539 bp homologous gene segment, comprising of five haplotypes that varied in abundance and geographical distribution. The most abundant haplotypes were haplotype 1 and haplotype 2, with haplotype 1 being found across all sample localities and haplotype 2 being found throughout the northern part of the island.

The determination of disease transmission through urine contamination was explored by molecularly screening the kidneys of the house mice for *Streptococcus* genome presence. A low *Streptococcus* prevalence of 7.4% was recovered. In order to validate the potential novel *Streptococcus* species reported previously, four culturing attempts were undertaken. Any bacterial growth recovered from the culture attempts were screened using a universal 16S primer set to identify the bacteria to genus level and where possible classify them to species level. Subsequent sequencing of PCR positive samples revealed $^{8}/_{9}$ samples to be novel and having the highest nucleotide sequence identity match (96%) to *S. constellatus*, while the remaining positive PCR sample grouped most closely to *S. parasanguinis* with 99% sequence similarity.

To determine *Helicobacter* prevalence, DNA was extracted from the gastrointestinal (GI) tracts as well as the livers of the mice and used as template for 16S rRNA lineage-specific amplification. The overall *Helicobacter* infection rate was 12.4%, with different infection rates being observed in the GI tracts (10.7%) and in the livers (2.5%). Two species were recovered *viz. H. typhlonius* and *H. hepaticus*, with the

remaining five samples appearing to contain novel *Helicobacter* species. Lastly, the presence of *Bartonella* was evaluated by targeting the livers of the mice and screening them with primers targeting the citrate synthase (*gltA*) gene. All 171 samples screened were negative, corresponding to an overall prevalence of 0%. These results contrast markedly with the generally high levels of prevalence reported for other murid rodent species.

In conclusion, *Mus musculus* from Marion Island harbour at least two genera with known zoonotic potential. However, the extent and severity of potential disease transmission risk that these invasive rodents pose to native vertebrates is not known and requires further investigation. Future studies should focus on a wider range of zoonotic bacteria and also take into consideration the potential effect of seasonality on disease transmission.

Keywords: House mouse, Marion Island, *Bartonella*, *Helicobacter*, *Streptococcus*, mitochondrial DNA

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CHAPTER 1

General introduction

(1.1) The study species, Mus musculus

Rodents form the most abundant and diversified order of living mammals, representing about 43% of all mammalian species (Huchon *et al.* 2002), with approximately 2277 species (Musser and Carleton 2005). Most rodents are environmentally beneficial as they form a food source for predators and act as a mechanism for seed dispersal; however, there are those few that are considered pests. An example of such a pest species is the house mouse (*Mus musculus*).

The house mouse (*Mus musculus* species complex) is believed to have originated from the Indian subcontinent (Boursot *et al*. 1996) and appears to have undergone a geographic radiation that started less than a million years ago (Boissinot and Boursot 1997). At least three different house mouse subspecies exist that occupy different geographical areas, with a western (*M. musculus domesticus*), a central (*M. musculus musculus*), and an eastern (*M. musculus castaneus*) house mouse species currently being recognized (Boissinot and Boursot 1997; Voolstra *et al.* 2007). Of the three well-known *Mus musculus* subspecies, *M. musculus domesticus* has been widely spread to numerous islands by European colonisation (Musser and Carleton 2005).

(1.2) Mus musculus *introduction to Marion Island*

Rodents, in particular the house mouse, have been introduced to more than two hundred oceanic Islands, including the sub-Antarctic islands of Antipodes, Gough, Kerguelen, Marion and St. Paul (Angel *et al.* 2008). *Mus musculus* was introduced by sealers to sub-Antarctic Marion Island in the early 1800's (Hänel and Chown 1999), and was found abundantly and widespread over the entire Island by 1818 (Watkins and Cooper 1986). Recent molecular studies have revealed that the mice introduced to Marion Island belong to the *Mus musculus domesticus* subspecies, with the close association of Marion Island haplotypes to haplotypes sampled from Porto Santo and northern Europe being indicative of a Scandinavian origin, or similarly introduced to

Marion Island through transport from the Madeira Archipelago (Van Vuuren and Chown 2007).

Mitochondrial DNA is widely used in population genetics, phylogeographical and phylogenetic studies as it provides easy access to an orthologous set of genes that experience little or no recombination and which undergo rapid evolution. This is useful as haplotype frequencies are governed primarily by migration and genetic drift (William *et al*. 2005). The mammalian mitochondrial DNA (mtDNA) genome is a circular double-stranded molecule that encodes 13 genes of the respiratory chain (Goios *et al.* 2007) and is involved in the mitochondrial translation apparatus, electron transport, and oxidative phosphorylation (William *et al*. 2005).

It is thought that the combined effect of feral cat removal in the 1990's (Bester *et al*. 2000) as well as climate change, has led to the mouse population increasing (Van Aarde *et al.* 1996). Climate change has the potential to increase mouse densities by creating longer breeding seasons for the mice as well as the invertebrates which they feed on, or by increasing the terrestrial productivity (Angel *et al.* 2009). The steady increase in the mouse population has been shown to have a significant impact on indigenous species (Van Vuuren and Chown 2007) as well as cause potential irreversible changes to ecosystem functioning on the island (Smith *et al*. 2002). This can also be seen by the extensive damage caused to plant communities particularly the cushion plant (*Azorella selago*) through the runways and extensive burrow systems of the mice (Avenant and Smith 2003).

The mice on Marion Island not only feed on endemic invertebrates but also on the seeds and young shoots of five native and one introduced plant species (Angel *et al.* 2009). In the 1960s the native sedge (*Uncinia compacta*) was found abundant in mire vegetation across Marion but has now almost been extirpated from this habitat due to seed predation by the mice (Smith and Steenkamp 1990). The mice are also thought to have a similar impact on the herbaceous *Acaena magellanica* (Avenant 1999). The importance of plant material compared to invertebrates in mouse diet has increased from an average of 36% in the late 1970s to 59% in the 1990s (Avenant 1999; Smith *et al.* 2002). The change in foraging behaviour of the mice is thought to be due to mouse predation reducing invertebrate densities (Crafford and Scholtz 1987).

(1.3) Mus *as reservoirs of zoonotic infection*

As the mouse population increases so do contact opportunities between this invasive species and the Island endemics, which in turn increases the potential of rodent-borne diseases spilling over to other native vertebrate species on the Island. Studies on *Mus musculus* reservoir host potential are limited to murine viruses on two Islands: Boullanger Island and Macquarrie Island (Moro *et al*. 2003), and to the assessment of three agents displaying host gender bias on Marion Island, *viz.* encephalomyocarditis virus, *Leptospira* and *Streptococcus* (de Bruyn *et al.* 2008).

It was recently proposed that a male-biased seal die-off that occurred at Marion Island in 2007 could have been precipitated by invasive mice (de Bruyn *et al*. 2008). Although no definitive link was found due to the absence of autopsy samples from the seals that succumbed, the report highlighted the possible threat posed by introduced mice as reservoirs of infection for native vertebrate species. In the de Bruyn *et al.* (2008) study it was proposed that seal rookeries contaminated with mouse urine provides a likely source of environmental contamination that facilitates the transmission of mouse-borne diseases. As kidneys generally have lower levels of bacterial species compared to the gut, identification of unknown bacteria using broad-range bacterial polymerase chain reaction (PCR) primers is possible (Nikkari *et al.* 2002). Rodents are reservoirs for many zoonotic bacteria; however, for the purpose of this study I will only be focusing on three *viz. Streptococcus*, *Helicobacter* and *Bartonella*.

(1.3.1) Streptococcus *and* Lactobacillus

Following on from a study conducted in 2008 (Eadie 2008) it was shown that simultaneous screening of mouse kidneys for *Lactobacillus* and *Streptococcus* is necessary as 24% of the 50 individuals assessed were found to be co-infected, resulting in mixtures when sequencing products generated with universal 16S primers.

Streptococci are a heterogeneous group of bacteria, consisting of as many as 48 species, including important human pathogens such as *Streptococcus pneumoniae*, *S. pyogenes*, and *S. agalactiae* (Facklam 2002). *Streptococcus pneumoniae* is a common agent of community-acquired pneumonia and endocarditis, whilst *S. agalactiae* is an

important cause of neonatal infections characterized by sepsis and meningitis (Picard *et al*. 2004). *Streptococcus pyogenes* causes a wide variety of serious infections including pharyngitis, soft-tissue infections, scarlet fever and rheumatic fever (Picard *et al*. 2004).

In inbred mice there is an influence of sex on the susceptibility toward streptococcal infection with increasing levels of testosterone leading to an increased susceptibility to a strain of *S. pyogenes* type 18 (Willoughby and Watson 1964). The occurrence of this bacterium in blood-rich organs along with the recent identification of a novel *Streptococcus* species in more than 30% of *Mus musculus* kidneys from Marion Island mice (de Bruyn *et al.* 2008), necessitated the screening of the mouse kidneys for presence of bacterial genomes.

Lactobacilli are considered to be nonpathogenic commensals of the human gut flora (Sun *et al.* 2007) and are beneficial to the intestinal flora (Tannock *et al.* 1989). The probiotic effects of lactobacilli stimulate the immune system and has resulted in the probiotic diary products for human consumption. These products are shown to enhance immune functions and thus reduce the risk of infection (Matsuzaki and Chin 2000).

Lactobacillus species colonize murine stomachs and intestine shortly after birth and then adhere to the epithelial cells to form part of the intestinal microbiota of the animals during development and adulthood (Schaedler *et al.* 1965). Lactobacilli are known to suppress the growth of other bacteria and some fungi (Savage 1969). Examples of bacteria that are suppressed in mice through lactobacilli persistence include *Helicobacter hepaticus* (Peña *et al.* 2005) and *Salmonella enterica enterica* serovar *typhimurium* (Moura *et al.* 2001).

(1.3.2) Helicobacter *in murid rodents*

The *Helicobacter* genus is characterized by two major evolutionary lineages with more than 30 species being described (Comunian *et al.* 2006). The two most common *Helicobacter* pathogens known to infect humans are *H. pylori* and *H. felis* (Fritz *et al.* 2006). Due to the widespread and persistent nature of *H. pylori*, a high proportion of humans are at risk of gastric tumours (Correa 1992). This bacterium has been shown to be invasive, colonising the surfaces of the gastric mucosa (Engstrand 2001).

A number of *Helicobacter* species are known to occur in rodents, particularly in mice. The *Helicobacter* species most commonly found in mice are *H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rappini*, *H. rodentium* and *H. typhlonius* (Whary and Fox 2006). A few of these *Helicobacter* species are pathogenic to mice, namely *H. hepaticus* and *H. bilis*, causing chronic active hepatitis and/or inflammatory bowel disease (Shomer *et al*. 1997; Ward *et al.* 1996); whilst the vast majority of infected animals display no clinical signs of infection (Whary and Fox 2006).

As *Helicobacter* occur in the mucosa and are passed on and transmitted via the faecal-oral route, tissue samples targeted for molecular analysis of *Helicobacter* should include the gastro-intestinal tract (GIT). Screening of liver samples is also advised, as hepatic forms of *Helicobacter* are known to occur (Fox *et al*. 1994; Patterson *et al*. 2000).

(1.3.3) Bartonella *in murid rodents*

The *Bartonella* genus is currently composed of 19 species (Gundi *et al.* 2009), of which almost half have known zoonotic potential. The genus consists of two human-specific pathogens, *Bartonella bacilliformis* and *Bartonella quintana*, which cause Carrion's disease and trench fever, respectively (Berglund *et al*. 2010). Infection with rodent *Bartonella* spp. such as *B. elizabethae* and *B. vinsonii* subsp. *arupensis* in humans can results in endocarditis and febrile illness, respectively (Daly *et al.* 1993, Welch *et al.* 1999). Factors known to influence *Bartonella* infection include genetic susceptibility, age-associated immunocompetence, and pregnancy; while congenital transmission could also contribute to a predilection of a given *Bartonella* species for infecting a specific host species more frequently (Breitschwerdt and Kordick 2000).

All *Bartonella* species are believed to be vector-transmitted, blood-borne, intracellular organisms with transmission being influenced by vector-host preferences (Breitschwerdt and Kordick 2000). Vectors responsible for *Bartonella* transmission include haematophagous invertebrates such as ticks (Welch *et al.* 1999), fleas (Chomel *et al*. 1996) and lice (Roux and Raoult 1999). Rodents generally have high levels of infection (Pretorius *et al.* 2004; Bastos 2007), and if occurring in close contact with humans represent a reservoir of infection (Breitschwerdt and Kordick 2000).

Bartonella is a fastidious bacterium which can take up to 6 weeks to culture making molecular-based methods for diagnosis more efficient (Agan and Dolan 2002). As *Bartonella* is an erythrocyte-infecting bacterium (Berglund *et al*. 2010), blood-rich organs such as the heart and liver are suitable for molecular screening of *Bartonella* genome presence.

(1.4) Rationale for and aims of the study

There is little known about bacterial reservoir host potential in *Mus musculus* on islands. This is very surprising in that the house mouse is the most geographically wide-spread species apart from humans (Musser and Carleton 2005). The de Bruyn *et al.* (2008) study highlighted the need to assess the disease risks posed by this invasive species to island endemics by emphasizing the potential for environmental contamination and inter-species transmission.

The primary aim of my study will be to molecularly determine the prevalence and diversity of three bacterial disease agents in the mice, all of which have a broad species recognition range namely: *Bartonella*, *Helicobacter* and *Streptococcus*. Any novel species discovered will be validated further through culturing and biochemical characterization. The secondary aim of my study will be to expand on the preliminary mitochondrial study on *Mus* conducted by Van Vuuren and Chown (2007). This will be accomplished by sampling more mice from a broader geographical range and by targeting a larger portion of the mitochondrial genome with the aim of more accurately determining the origin of the introduced mice, evaluating genetic diversity and mapping their dispersal on the island.

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CHAPTER 2

Mitochondrial sequence diversity of the house mouse, *Mus musculus domesticus*, introduced to sub-Antarctic Marion Island

Abstract

The house mouse (*Mus musculus domesticus*) was introduced to sub-Antarctic Marion Island in the early 1800's by sealers. It is a highly established alien invasive species that has a negative impact on the indigenous fauna and flora, regardless of habitat type. Little is known about this invasive species regarding its genetics and infectious disease potential. As a first step in addressing host genetics, the genetic diversity of a sub-population of mice, comprising of 46 individuals, was evaluated by mitochondrial DNA sequence analysis. Amplification of ~ 1.7 kbp fragment inclusive of cytochrome *b* (cyt *b*), tRNA-Thr, tRNA-Pro and the mitochondrial DNA control region was amplified and sequenced for all 46 specimens from seven localities. As no sequence variation was recovered in cyt*b* and the tRNA-Thr and tRNA-Pro regions, subsequent sequencing efforts were directed exclusively at the control region, resulting in a 539 bp dataset comprised of 91 individuals collected from seven geographically separate localities on Marion Island. Five haplotypes were recovered that varied in abundance and geographical distribution. Nucleotide sequence comparison to homologous data in the Genbank database, confirmed the finding of a previous study that linked Marion Island mice to *Mus musculus domesticus* collected from Porto Santo, Madeira.

Keywords: House mouse; Marion Island, mitochondrial DNA and geographical distribution.

Introduction

Mitochondria are thought to have originated from free-living, aerobic, and motile α-proteobacteria (Boussau *et al.* 2004). The mammalian mitochondrial DNA (mtDNA) genome is a circular double-stranded molecule that encodes 13 genes of the respiratory chain (Goios *et al.* 2007) and is involved in the mitochondrial translation apparatus, electron transport, and oxidative phosphorylation (William *et al*. 2005). Mitochondrial DNA is widely used in population genetics, phylogeographical and phylogenetic studies as it provides easy access to an orthologous set of genes that experience little or no recombination and rapid evolution. This is useful as most variation within a species is selectively neutral and haplotype frequencies are governed primarily by migration and genetic drift (William *et al*. 2005).

The house mouse (*Mus musculus* species complex) is believed to have originated from the Indian subcontinent (Boursot *et al*. 1996), and appears to have undergone a geographic radiation that started less than a million years ago (Boissinot and Boursot 1997). At least three different house mouse subspecies exist that occupy different geographical areas, *viz.* a western (*M. musculus domesticus*), a central (*M. musculus musculus*), and an eastern (*M. musculus castaneus*) house mouse subspecies (Boissinot and Boursot 1997; Voolstra *et al.* 2007).

Mus musculus is believed to have been introduced by sealers to sub-Antarctic Marion Island in the early 1800's (Hänel and Chown 1999), and was found abundantly and widespread over the entire Island by 1818 (Watkins and Cooper 1986). It is thought that the combined effect of feral cat removal in the 1990's (Bester *et al*. 2000) as well as climate change, has led to the mouse population increasing (Van Aarde *et al.* 1996). This steady increase has been shown to have a significant impact on indigenous species as well as ecosystem functioning on the island (Van Vuuren and Chown 2007). This can also been seen with the extensive damage caused to plant communities particularly the cushion plant (*Azorella selago*) through the runways and extensive burrow systems of the mice (Avenant and Smith 2003).

A recent molecular study revealed that the mice on Marion Island belong to the *Mus musculus domesticus* subspecies and with the close association of Marion Island

haplotypes to haplotypes sampled from Porto Santo and northern Europe being indicative of a Scandinavian origin, or similarly introduced to Marion Island through transport from the Madeira Archipelago (Van Vuuren and Chown 2007).

This study aims to expand on this preliminary report by sampling more mice from a broader geographical range and by targeting a larger portion of the mitochondrial genome with the aim of obtaining a more accurate determination of the origin of the introduced mice, and to map their dispersal on the island, should the genetic diversity be sufficiently high to allow for this.

Materials and methods

Sampling

The study site was the sub-Antarctic Marion Island (290 km^2) situated at 46°54'S and 37°45'E, which is situated approximately 2180 km southeast of Cape Town, South Africa (Pistorius *et al*. 2001). The mice were collected from Marion Island with permission from the Department of Environmental Affairs and Tourism, and with the approval of the Prince Edward Islands Management Committee. The mice were sampled from seven sites across Marion Island, namely Base, Cape Davis, Kildalkey, Mixed pickle, Repettos, Swartkops and Watertunnel (Fig. 1).

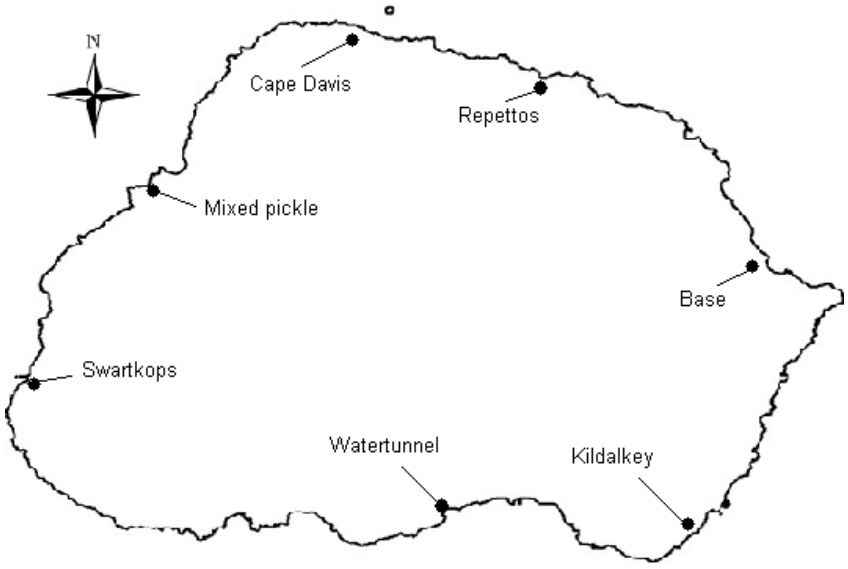

Fig. 1 Sampling localities for mice captured during 2009 take over on Marion Island.

Of the 121 mice caught, 91 specimens representative of all localities sampled were used for this host genetics characterization component of the study. To determine the geographic origin of these mice, mtDNA sequences of seventeen *Mus musculus domesticus* sequences were downloaded from Genbank (Table 1). Reference strains of eighteen *Mus musculus domesticus* sequences from sub-Antarctic Kerguelen Archipelago (Hardouin *et al*. 2010) were also included to assess haplotype variability (Table 1), with two *Mus musculus musculus* sequences (Genbank accession numbers HQ241755 and HQ241756) being used as an outgroup (Smith 1994; Dalevi *et al*. 2001; Huelsenbeck *et al*. 2002). These mtDNA sequences were selected to provide geographic coverage of the global distribution of this subspecies.

Table 1 *Mus musculus domesticus* geographic origin, Genbank accession numbers of the sequences and the abbreviations used in this study.

Geographic origin	**Abbreviations used for network**	**Accession number**	**Reference**
Cameroon	Cam	AM182714	Ihle *et al.* 2006
France	Fra1	AM182736	Ihle *et al.* 2006
France	Fra2	AM182742	Ihle *et al.* 2006
Germany	Ger1	FJ374655	Yu *et al.* 2009
Germany	Ger2	GQ871746	Unpublished
Greece	Gre1	AY551946	Tryfonopoulos *et al.* 2005
Greece	Gre2	AY551961	Tryfonopoulos *et al.* 2005
Iran	Iran	AJ286320	Gündüz *et al.*2000
Italy	Ita1	AY560832	Castiglia *et al.* 2005
Italy	Ita2	U47471	Prager *et al.* 1996
Madeira	Mad1	GQ242002	Förster *et al.* 2009
Mauritania	Mau	AJ313380	Gündüz *et al.*2001
Morocco	Mor	AJ313381	Gündüz *et al.*2001
Porto Santo Island, Madeira	Mad2	AJ313379	Gündüz *et al.*2001
Portugal	Por	GQ242020	Förster *et al.* 2009
Turkey	Tur1	AJ843827	Gündüz *et al.*2005
Turkey	Tur2	AJ843833	Gündüz *et al.*2005
French Southern and Antarctic	Ams	HQ185258	Hardouin *et al.* 2010

Lands: Amsterdam Island			
Kerguelen Archipelago: Cimetiere Island	Cim	HQ185259	Hardouin *et al.* 2010
Falkland Islands: New Island	New1	HQ185260	Hardouin *et al.* 2010
Falkland Island: New Island	New2	HQ185261	Hardouin *et al.* 2010
Falkland Islands: East Cape Pembroke	ECP	HQ185262	Hardouin *et al.* 2010
Falkland Islands: West Bold Cove	WBC	HQ185263	Hardouin *et al.* 2010
Falkland Islands: West Port Stephens	WPS	HQ185265	Hardouin *et al.* 2010
Falkland Islands: Saunders Island	SI	HQ185266	Hardouin *et al.* 2010
Falkland Islands: Steeple Jason Island	SJI	HQ185269	Hardouin *et al.* 2010
South Georgia	SGe	HQ185272	Hardouin *et al.* 2010
Kerguelen Archipelago: Port-Couvereux	Pcou	HQ185273	Hardouin *et al.* 2010
Kerguelen Archipelago: Jacky	Jac1	HQ185275	Hardouin *et al.* 2010
Kerguelen Archipelago: Jacky	Jac2	HQ185276	Hardouin *et al.* 2010
Kerguelen Archipelago: Mayes Island	May	HQ185277	Hardouin *et al.* 2010
Kerguelen Archipelago: Port-aux francis	PAF	HQ185278	Hardouin *et al.* 2010
Kerguelen Archipelago: Port-Jeanne d'Arc	PJA1	HQ185280	Hardouin *et al.* 2010
Kerguelen Archipelago: Port-Jeanne d'Arc	PJA2	HQ185281	Hardouin *et al.* 2010
France	Fra3	HQ185282	Hardouin *et al.* 2010

DNA extraction, amplification and sequencing

A fragment of tissue from the liver of each mouse was dissected out, placed in individual 1.5 ml eppendorf tubes on ice and transferred to a -20°C freezer for storage. DNA was extracted from the livers using the Roche high Pure PCR template preparation

kit (Roche Applied Science) following the manufacturer's prescribed protocol for nucleic acid extraction from mammalian tissue.

Mitochondrial genome amplification was achieved using genus-specific primers (Table 2) targeting ~1.7kbp fragment of the cyt *b* and control region. Standard polymerase chain reactions (PCRs), employing cyt *b* and control region primers L14086-MAB and H15870-MAB designed specifically for this study on the basis of available *Mus* sequences in Genbank, were used in conjunction with a step-down thermal cycling profile consisting of an initial denaturation step at 96°C for 20s followed by 2 cycles of denaturation at 96°C for 12s, annealing at 56°C for 25s, and extension of 72°C for 2min; followed by 3 cycles of denaturation at 96°C for 12s, annealing at 54°C for 20s, and extension of 72°C for 1min 50s; and finally 35 cycles of denaturation at 96°C for 12s, annealing at 52°C for 15s, and extension of 72°C for 1min 40s.

PCR product size was estimated by 1.5% agarose gel electrophoresis of 5µl of the product, against a 1kb size standard (GeneRuler, Fermentas) ladder. Negative and positive controls were included to preclude false positives due to reagent contamination, and false negatives, respectively. All positive PCRs were purified from the tube using a Roche PCR product purification kit (Roche, Mannheim Germany) according to manufacturer's specifications. Cycle-sequencing reactions were performed at primer-specific annealing temperatures (summarised in Table 2) using Big Dye Terminator Cycle Sequencing Kit version 3.1 (Perkin-Elmer, Foster City, USA), with each of the appropriate external PCR primers, and run on an AB3130. The internal primer L15251-Rev was used to generate control region sequences. The first 46 samples were sequenced with all three primers and the resulting overlapping sequences were used to generate a contiguous 1.7kb sequence for each specimen. As no sequence variation was recovered for the cyt*b* region, subsequent sequencing was performed with the control region primers, L15251-Rev and H15870-MAB. Sequence chromatograms were viewed, edited and aligned in the Chromas programme included in MEGA4 (Tamura *et al.* 2007). Each nucleotide sequence was then blasted against the Genbank database (www.ncbi.nlm.nih.gov/blast) to confirm the *M. m. domesticus* sub-species designation.

Table 2 Primers designed specifically for this study detailing the binding position in the *Mus* mtDNA genome, melting temperatures (Tm) and their use.

Primer Name	**Primer sequence**	**Binding position**	**Tm (GC content)**	**Primer use**
L14086-MAB	GCATTCAACTGCGACCAATGA	14086- 14106	58°C (10/21)	PCR & sequencing
L15251-Rev	CTCAGGAATTATCGAAGACA	15251-15270	53°C (8/20)	Sequencing
H15870-MAB	CCGATACCATCGAGATGTCT	15870-15851	57°C (10/20)	PCR & sequencing

Data analyses

All generated sequences were initially aligned to the reference sequences downloaded from the Genbank database using the ClustalW programme incorporated in MEGA4 (Tamura *et al*. 2007), and adjusted manually to further improve the alignment. Basic sequence statistics including the proportion of variable and parsimony informative sites, as well as base composition and the transition:transversion ratio (R), were estimated in MEGA4. JModelTest v0.1 (Posada 2008) was used to estimate the model of evolution that best fitted the dataset. The best-fit model selected under the Akaike Information Criterion (AIC) was used for all phenetic analyses.

Neighbour-joining (NJ) and minimum evolution (ME) trees were inferred using pairwise deletion as well as complete deletion of gaps and missing data. This was done to evaluate the effect of differential treatment of both gaps and missing data on the resultant phylogenies. As there was no difference in the trees produced, pairwise deletions were used to accommodate missing data/gaps in the dataset. To evaluate the influence of parameter complexity on tree topology, an uncorrected *p*-distance tree was compared to a tree inferred with the Tamura-Nei model of sequence evolution in MEGA4. As no topological differences were noted, a ME tree inferred on the basis of observed, uncorrected changes, is presented (Fig. 2), with bootstrap values obtained from phenetic analyses with the best-fit model of evolution selected under the Akaike Information Criterion (AIC) in JModelTest v0.1 (Posada 2008), being transferred to the corresponding nodes.

Bayesian Inference (BI) analyses were preformed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2006), and made use of AIC-selected best-fit model priors from JModelTest v0.1 (Posada 2008). The Markov chain Monte Carlo (MCMC) process was started from random starting points with four chains being run simultaneously for 10 000 000 generations, using default heating and swap parameters. Trees and parameters were recorded every 100 generations and the first 25 000 trees (25 %) were discarded as the 'burn-in'. Two independent runs were preformed to ensure that the results converged.

Network, a program using median-joining and which considers multi-state data (Forster *et al.* 2000) was used to infer the population level genealogies (Clement *et al.* 2000), presented in figure 3. Nucleotide and haplotype diversity was calculated using the program DnaSP v. 5.0 (Librado and Rozas 2009). The geographical distribution of haplotypes is presented in figure 4.

Results

Haplotype diversity

The amplification of the mitochondrial DNA control region generated a 539 bp product for 91 Marion Island specimens and resulted in the recovery of 5 haplotypes. Four haplotypes, including two previously published Marion haplotypes, 1 and 2 (Van Vuuren and Chown 2007), characterized the sixteen specimens sampled at Repettos. The only haplotype to be found at all the sample localities was haplotype 1 (fig. 4). This haplotype was notably the most abundant haplotype occurring in $^{65}/_{91}$ mice sampled (fig 4). A summary of the sex, sampling locality and haplotypes recovered for each individual *Mus musculus* is summarised in Appendix A.

The Network program is useful as it reconstructs phylogenetic networks using the median-joining algorithm which compensates for multi-state data and ambiguities. However should an ambiguity occur at the beginning of a sequence then it can be mistaken for a mutation. This is relevant as some of the reference sequences were shorter than those generated in this study making it necessary to reduce the size of the global dataset which incorporated data from previous studies. This resulted in the loss of one of the haplotypes found on Marion (haplotype 5) because of a lack of available homologous

data in the variable region defining this haplotype. The final dataset used for the global Network analysis was 378 bp in length and contained four Marion Island haplotypes, thirty five reference sequences and contained a total of thirty one haplotypes.

From the 539 bp Marion-only dataset, nucleotide diversity pairwise as well as individual sites comparison was 0.00085 (SD=0.00012). Five haplotypes were recovered using DnaSP v5.0, with the haploype diversity being 0.449 and the variance of haplotype diversity being 0.00286 (SD=0.053).

Phylogenetic analyses

Due to reference sequences being shorter than the sequences obtained in this study, the global dataset was reduced to 378 bp of homologous data to ensure that there was no missing data. The dataset comprised of 351 (92.9%) conserved, 27 (7.1%) variable, 16 (4.2%) parsimony informative and 11 (2.9%) singleton sites. The TIM1+I+G model selected under the Akaike information criterion in JModelTest v0.1 (Posada 2008) had a proportion of invariable sites (I) of 0.7910 and a gamma distribution shape parameter (G) of 0.5520 which was used for BI and ML analyses. The estimated mean base frequencies under this model were A = 0.3221, C = 0.2517, G = 0.1162 and T = 0.3100. Nodal support was assessed by 5000 bootstrap (BS) replicates for ML and 10000 BS for ME. In order to reduce computation time, all identical sequences were excluded from the dataset for all analyses, resulting in a final dataset containing 41 taxa inclusive of the four representative Marion Island haplotypes.

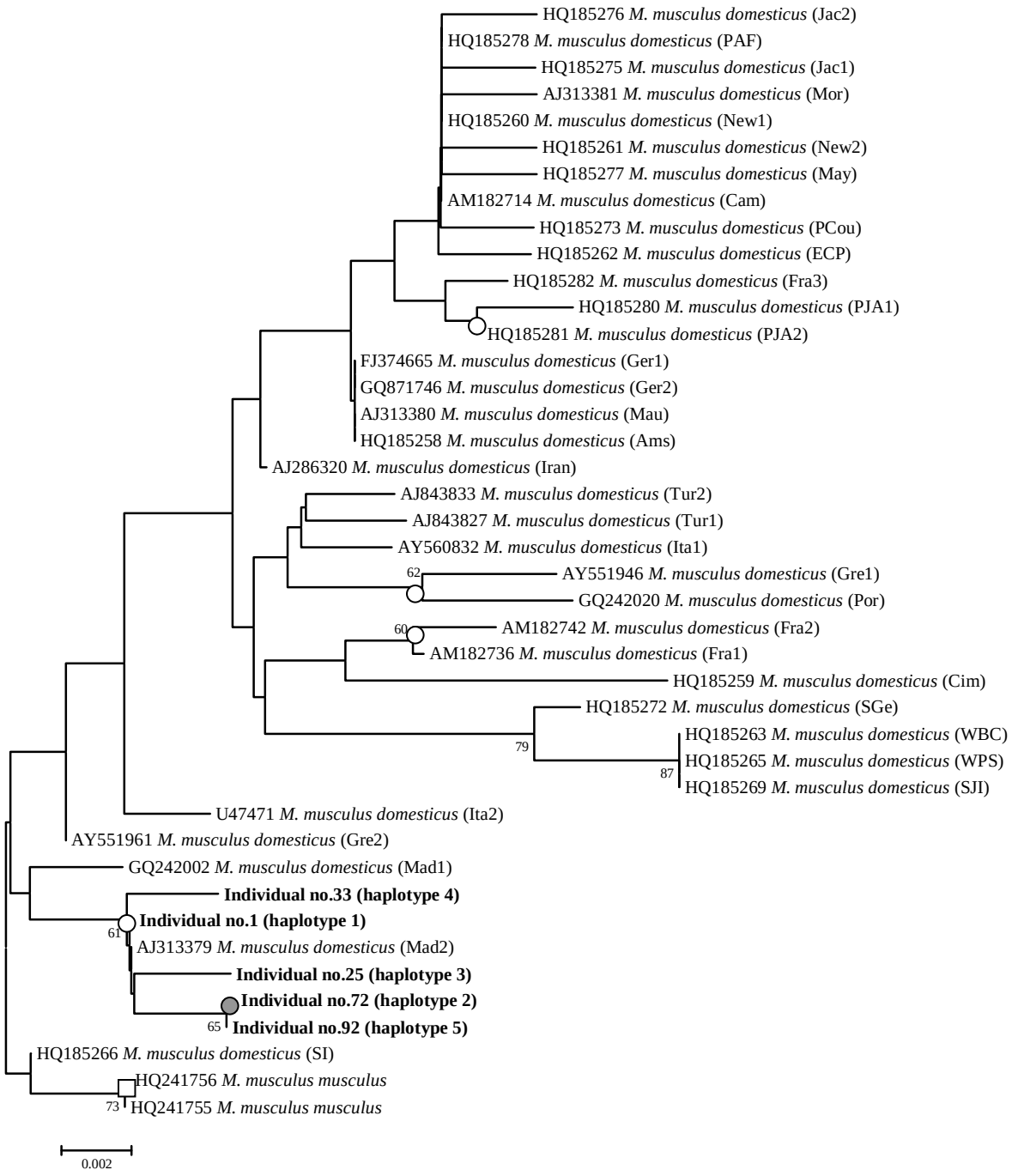

Fig. 2 A 50 % majority rule consensus tree of the D-loop gene region inferred with Minimum Evolution (ME) using pairwise deletion of gaps and missing data, and the Tamura-Nei + G (0.5520) model of sequence evolution. Bootstrap support values shown are those ≥ 50 obtained from 10,000 pseudoreplicates. Nodes that had high levels of support in both parsimony and Bayesian analyses are indicated as follows: open circles: 51-65 %, closed circles: 66-75 %, open squares: 85-95 % and closed squares: 96-100 %. Taxon names for reference sequences comprise of the Genbank accession numbers, with the abbreviations used in Network indicated in brackets. Samples characterized in this study are indicated in bold.

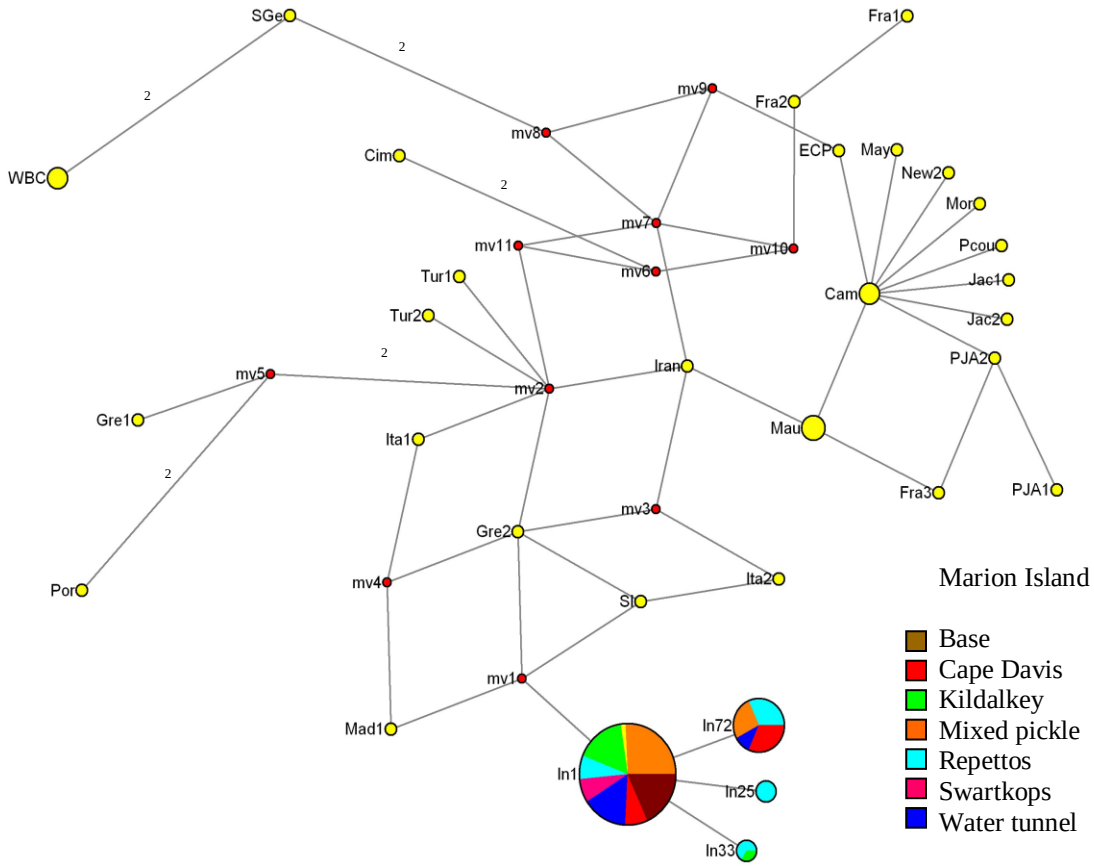

Fig. 3 D-loop haplotype network generated using Median Joining for *Mus musculus domesticus* samples with reference samples indicated in yellow and the size of the circles representing the frequency of each haplotypes on Marion Island. Each node is one mutational step away from the next node with numbers indicating where more than one mutation was required to join the nodes. The median vectors indicated by the small red circles represent branch splits or potential missing haplotypes needed to connect the nodes. The three letter country/locality codes are those provided in Table 1 and the Marion Island haplotypes are as follows: In1 (haplotype 1), In72 (haplotypes 2), In25 (haplotype 3), In 33 (haplotype 4), with the yellow pie segment in In1 corresponding to Madeiran reference haplotype / sequence 'Mad2'.

A consistent finding, irrespective of the method of analyses used, is that the Marion Island haplotypes were most similar to the haplotype sampled on Porto Santo (Mad2) within the Madeira Archipelago, with the most abundant haplotype, (Marion haplotype 1, represented by Individual 1 and In1 in the tree and network, respectfully) being identical to Mad2 (AJ313379) across the control gene region characterized in this study. From the

Network analysis it is evident that the Marion samples are most closely related to the Madeira sample (Mad2) used in the Gündüz *et al*. (2001) study, as it grouped within the haplotype 1 (In1) cluster. The Marion sample In1 (haplotype 1) next grouped most closely to Madeira (Mad1), Greece (Gre2) and Saunders Island (SI) samples, with only two mutational steps difference.

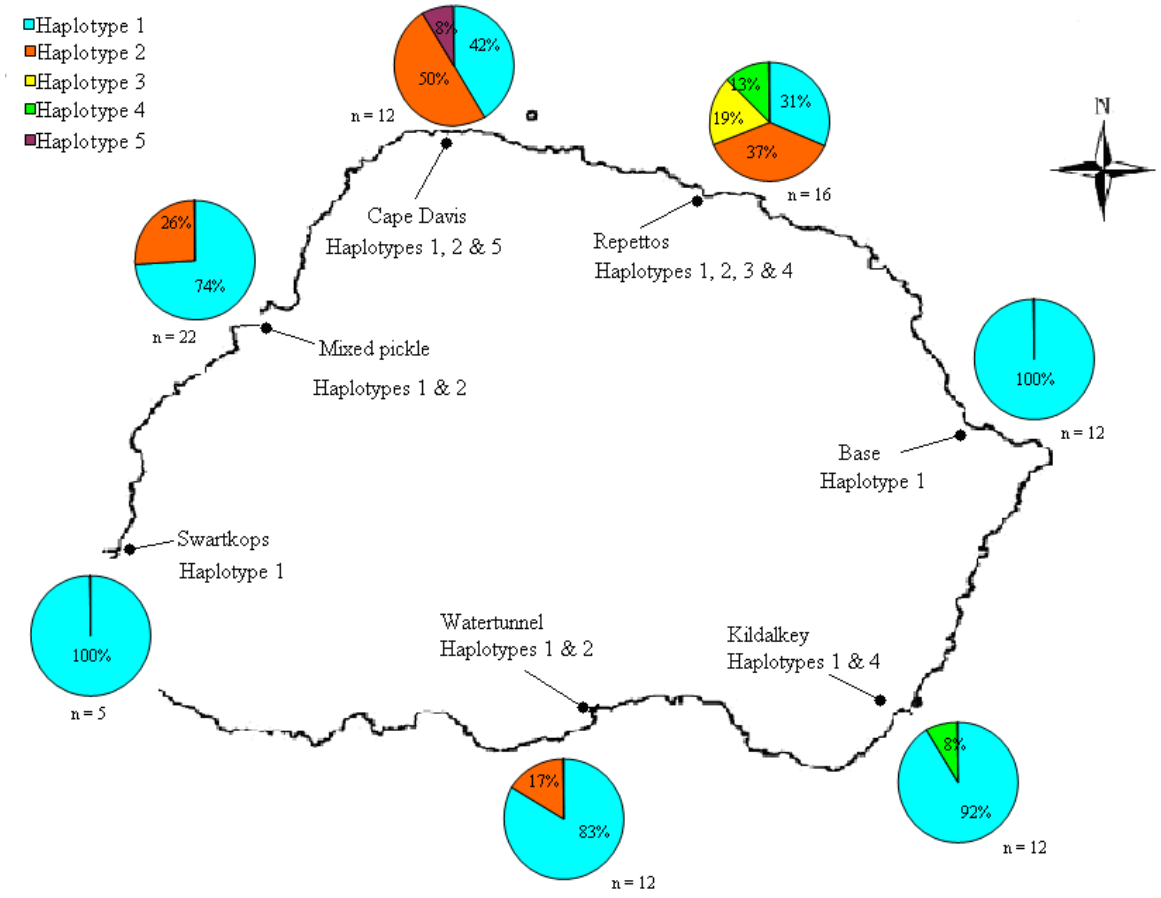

Fig. 4 Geographical distribution of the haplotypes obtained at Marion Island with the pie charts representing the proportion of haplotypes obtained for the respective sampling localities, with the sample size (n) of individuals used indicated next to each pie chart. Haplotypes 1 and 2 correspond to the Marion haplotype 1 and Marion haplotype 2, which were first described in the study Van Vuuren and Chown (2007) in which 10 individuals from two localities (Base & Mixed Pickle) were characterised.

Discussion

House mice were believed to have been introduced in the early 1800's to Marion Island by sealers that came from Europe (including the United Kingdom and

Scandinavia) and North America (Hänel and Chown 1999). This study aimed to determine the origin of these invasive mice with greater accuracy than the Van Vuuren and Chown (2007) study, in which 10 mice sampled from Base (n = 8) and Mixed Pickle (n = 2) were genetically characterized. By increasing the sample size and sampling across a broader geographical area, a more accurate assessment of genetic diversity and distribution would be possible and indeed resulted in the recovery of three more haplotypes than reported previously. Further, a single, widespread and abundant haplotype (see fig. 4) was identified which most likely corresponds to the founder haplotype. From the and 100% sequence identity between this haplotype and a haplotype from Porto Santo Island, Madeira and from the one-step mutations between haplotype 1 and each of the four remaining Marion Island haplotypes, we can say with greater confidence that the mice present on Marion Island today originated from a single introduction. From figure 4 it is evident that the haplotype abundance and diversity differs greatly across Marion Island. In particular, haplotype diversity is greatest at Repettos suggesting that this locality is most likely the site of the original introduction. An introduction / invasion from the north is further supported by the high levels of haplotype diversity occurring at neighbouring Cape Davis.

A recent study of *Mus* diversity on islands recently invaded by this rodent genus (Hardouin *et al.* 2010) indicates that inclusion of nuclear genes for instance could provide more genetic variability which might give better resolution to where these mice originate from. William *et al.* (2005) also suggest that one should be cautious when recognizing genealogical species using only mtDNA, as the substitution rates could be influenced by thermal environment, with species in colder environments having either fewer or more mutations than species in warmer climates, depending on the species. The authors further warn that mtDNA has the potential for becoming monophyletic through selective sweeps. Despite the little genetic variation present in these invasive house mice, the mice were able to establish large populations across the Island regardless of habitat type.

The identical association of the Marion Island haplotype 1 to those sampled on Porto Santo, Madeira indicates that this northern hemisphere island which contains mice of Scandinavian origin, was the source for house mice on Marion Island. The mice were most likely introduced to Marion Island by sealers through transport from the Madeira

Archipelago (Van Vuuren and Chown 2007). Gündüz *et al.* (2001) argues that although the Madeiran Archipelago has been linked to Portugal, northern Europeans must have visited the islands long before the first Portuguese settlers. It has been documented that Danish Vikings made several raiding expeditions along the Iberian coast into the western Mediterranean (Gündüz *et al.* 2001). Future studies should include nuclear DNA when inferring genealogies and consider the function of mitochondria and its potential effects on organismal fitness when trying to infer evolutionary histories (William *et al.* 2005).

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Appendix A. Summary of the sex of individual *Mus musculus* mice as well as sampling locality and mtDNA haplotypes recovered on Marion Island.

Individual no.	Sampling locality	Sex	Haplotype recovered
1	Kildalkey	Male	Haplotype 1
2	Kildalkey	Male	Haplotype 1
3	Kildalkey	Female	Haplotype 1
4	Kildalkey	Female	Haplotype 4
5	Kildalkey	Female	Haplotype 1
6	Kildalkey	Male	Haplotype 1
7	Kildalkey	Male	Haplotype 1
8	Kildalkey	Female	Haplotype 1
9	Kildalkey	Male	Haplotype 1
10	Kildalkey	Male	Haplotype 1
11	Kildalkey	Male	Haplotype 1
12	Kildalkey	Female	Haplotype 1
19	Repettos	Female	Haplotype 2
20	Repettos	Male	Haplotype 4
21	Repettos	Male	Haplotype 1
22	Repettos	Female	Haplotype 2
23	Repettos	Male	Haplotype 1
24	Repettos	Male	Haplotype 2
25	Repettos	Female	Haplotype 3
26	Repettos	Female	Haplotype 3
27	Repettos	Female	Haplotype 2
28	Repettos	Male	Haplotype 2
29	Repettos	Male	Haplotype 1
30	Repettos	Female	Haplotype 1
31	Repettos	Male	Haplotype 1
32	Repettos	Male	Haplotype 2
33	Repettos	Male	Haplotype 4
34	Repettos	Male	Haplotype 3
35	Swartkops	Male	Haplotype 1
36	Swartkops	Female	Haplotype 1
37	Swartkops	Male	Haplotype 1
38	Swartkops	Male	Haplotype 1
39	Swartkops	Male	Haplotype 1
40	Watertunnel	Male	Haplotype 1
41	Watertunnel	Male	Haplotype 1
42	Watertunnel	Male	Haplotype 1
43	Watertunnel	Male	Haplotype 2
44	Watertunnel	Female	Haplotype 1
45	Watertunnel	Female	Haplotype 1
46	Watertunnel	Male	Haplotype 1
47	Watertunnel	Male	Haplotype 1

48	Watertunnel	Male	Haplotype 1
49	Watertunnel	Male	Haplotype 1
50	Watertunnel	Female	Haplotype 2
51	Watertunnel	Male	Haplotype 1
52	Mixed pickle	Female	Haplotype 1
53	Mixed pickle	Female	Haplotype 1
54	Mixed pickle	Female	Haplotype 1
55	Mixed pickle	Male	Haplotype 1
56	Mixed pickle	Female	Haplotype 1
57	Mixed pickle	Male	Haplotype 1
58	Mixed pickle	Female	Haplotype 1
59	Mixed pickle	Male	Haplotype 1
60	Mixed pickle	Male	Haplotype 1
61	Mixed pickle	Female	Haplotype 1
62	Mixed pickle	Male	Haplotype 1
63	Mixed pickle	Male	Haplotype 1
72	Mixed pickle fur seal peninsula	Female	Haplotype 2
73	Mixed pickle fur seal peninsula	Female	Haplotype 2
74	Mixed pickle fur seal peninsula	Male	Haplotype 2
75	Mixed pickle fur seal peninsula	Female	Haplotype 1
76	Mixed pickle fur seal peninsula	Male	Haplotype 1
77	Mixed pickle fur seal peninsula	Female	Haplotype 2
78	Mixed pickle fur seal peninsula	Male	Haplotype 2
79	Mixed pickle fur seal peninsula	Female	Haplotype 1
80	Mixed pickle fur seal peninsula	Female	Haplotype 1
81	Mixed pickle fur seal peninsula	Female	Haplotype 1
82	Cape Davis	Female	Haplotype 2
83	Cape Davis	Female	Haplotype 2
84	Cape Davis	Male	Haplotype 2
85	Cape Davis	Male	Haplotype 2
86	Cape Davis	Male	Haplotype 1
87	Cape Davis	Female	Haplotype 2
88	Cape Davis	Female	Haplotype 1
89	Cape Davis	Male	Haplotype 1
90	Cape Davis	Male	Haplotype 1
91	Cape Davis	Male	Haplotype 1
92	Cape Davis	Female	Haplotype 5
93	Cape Davis	Male	Haplotype 2
102	Base	Male	Haplotype 1
103	Base	Male	Haplotype 1
104	Base	Male	Haplotype 1
105	Base	Male	Haplotype 1
106	Base	Male	Haplotype 1
107	Base	Male	Haplotype 1
108	Base	Male	Haplotype 1

109	Base	Male	Haplotype 1
100	Base	Female	Haplotype 1
111	Base	Male	Haplotype 1
112	Base	Male	Haplotype 1
113	Base	Male	Haplotype 1

CHAPTER 3

The role of *Mus musculus* in contributing to environmental bacterial contamination on Marion Island

Abstract

Mice can transmit disease directly through biting, contamination of food and drinking water through urine and faeces or indirectly through their ectoparasites. For the purpose of this study, the potential for environmental contamination and transmission *via* the urinary route was investigated. To this end, kidney samples from 121 *Mus musculus* specimens sampled from seven geographically discrete localities across sub-Antarctic Marion Island were assessed to determine the prevalence of two bacterial genera previously shown to be harboured in the kidneys of these invasive rodents (de Bruyn *et al*. 2008). DNA extracted from kidneys of house mice were used as a template for *Lactobacillus* and *Streptococcus* genome screening. When using a genus-specific primer, an overall *Streptococcus* infection rate of 7.4% was recovered.

Keywords: Environmental contamination, *Streptococcus*, *Mus musculus*, Marion Island

Introduction

Rodents are responsible for enormous losses to food stores either by directly eating the foods or rendering them inedible through urine and faecal contamination. Mice can transmit disease in a number of ways including biting, contamination of food and drinking water through urine and faeces or indirectly through their haematophagous invertebrates (Meerburg *et al*. 2009). According to the Centre for Disease Control and prevention (CDC), the most common diseases transmitted by rodents are Weil's disease, Hantavirus Pulmonary syndrome, Haemorrhagic fever with renal syndrome, Lassa fever,

Leptospirosis, Lymphotic Chorio-menigitis (LCM), Omsk Haemorrhagic fever, Plague, Rat-bite fever, Salmonellosis, South American Arena virus and Tularemia (www.cdc.gov/rodents/diseases/direct.html)

Lactobacillus

Members belonging to the *Lactobacillus* genus can be found in a variety of habitats including fermented foods, silage and the digestive tracts of many animals (Rodtong and Tannock 1993). Lactobacilli are considered to be non-pathogenic commensals of the human gut (Peña *et al*. 2004; Sun *et al.* 2007) and are generally beneficial to the intestinal flora (Tannock *et al.* 1989). The commensal bacteria found in the intestines play an important role metabolizing digesta in the lumen; which provides an additional source of nutrients (Matsuzaki and Chin 2000). Probiotics are classified as the live microbes that benefit a host by improving the gastrointestinal microbial balance (Fuller 1989). The probiotic effects of lactobacilli stimulates of the immune system (Moura *et al.* 2001) and has resulted in the production of probiotic diary products for human consumption; which are shown to enhance immune functions and thus reduce the risk of gastrointestinal infection (Matsuzaki and Chin 2000) and inflammatory bowel disease (Madsen 2001; Macfarlane and Cummings 2002).

Lactobacillus species colonize murine stomachs and intestine shortly after birth, then adhere to the epithelial cells to form part of the intestinal microbiota of the animals during development and adulthood (Schaedler *et al.* 1965) and have been shown to be the numerically predominant bacteria detected in the proximal digestive tracts (Tannock *et al*. 1989). Matsuzaki and Chin (2000) showed that *Lactobacillus casei* (strain Shirota), not only stimulates the innate immune responses in rodents but also exerts potent antitumor and antimetastatic effects. Lactobacilli are known to suppress the growth of other bacteria and some fungi (Savage 1969). Examples of bacteria that are suppressed in mice through lactobacilli persistence include *Helicobacter hepaticus* (Peña *et al.* 2004), *Helicobacter pylori* (Aiba *et al*. 1998) and *Salmonella enterica enterica* serovar *typhimurium* (Moura *et al.* 2001).

Streptococcus

Streptococcus is a round, non-sporulating, gram-positive coccus that occurs in pairs or long chains (Kawamura *et al.* 1998; Lawson *et al.* 2005a). These bacteria belong to the phylum Firmicutes, class Bacilli, order Lactobacillales and the family Streptococcaceae. The *Streptococcus* genus is divided into 20 serogroups, also known as Lancefield groups A-V (with I and J being unused), and these serogroups are based on the extraction of cell wall surface antigens with hot hydrochloric acid (Tomida *et al.* 2011). To differentiate between the different streptococci certain characteristics and methods have to be considered including, colony size, haemolytic activity and phenotypic tests (fermentation ability and tolerance tests) (Sherman 1937; Tomida *et al.* 2011). Köhler (2007) proposed that the *Streptococcus* genus be divided into six phylogenetic groups namely anginosus, bovis, mitis, mutans, pyogenic and salivarius.

Streptococci are a heterogenous group of bacteria, consisting of as many as 60 species (Lawson *et al.* 2005b; Bekal *et al*. 2006; Tomida *et al.* 2011), including important human pathogens such as *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae* (Facklam 2002) and *S. iniae* (Weinstein *et al.* 1997). *Streptococcus pneumoniae* is a common agent of community-acquired pneumonia, bacteremia (Arbique *et al*. 2004) and meningitis (Echchannaoui *et al.* 2002); while *S. agalactiae* is an important cause of neonatal infections characterized by sepsis and meningitis (Schuchat 1998; Picard *et al*. 2004). *Streptococcus pyogenes* is the most pathogenic bacterium within the genus (Facklam 2002) and is known to cause a wide variety of serious infections including pharyngitis, soft-tissue infections, scarlet fever, rheumatic fever and toxic shock syndrome in humans (Picard *et al*. 2004; Medina and Lengeling 2005). Although *S. iniae* is primarily isolated in fish it can also cause bacteremia and meningitis in humans (Weinstein *et al*. 1997).

In inbred mice there is an influence of sex on the susceptibility of *S. pyogenes* type 18 infection with increasing levels of testosterone (Willoughby and Watson 1964; Medina *et al*. 2001). Medina *et al*. (2001) postulated that the observed sex differences in *S. pyogenes* resistance could either be attributed to X chromosome encoded resistance loci or to autosomal loci that are sex restricted in expression.

For the purpose of this study we will be focussing on the potential for bacterial transmission through possible urine contamination. In order to do this mouse kidneys were screened with *Lactobacillus*, *Streptococcus* and broad range bacterial universal 16S primers. PCR is a diagnostic tool that uses preselected, species-specific DNA regions for the genotypic identification of bacteria (Alber *et al*. 2004). The advent of PCR is not only specific and sensitive but can be reliable when using a range of templates including urine and renal tissue (Cameron *et al.* 2008); which is advantageous when dealing with the identification of fastidious bacteria. In addition to the molecular approach in which the 16S rRNA gene was targeted, a culturing approach was also undertaken to validate any potential novel species found.

Materials and methods

Sampling

The mice were collected from Marion Island with permission from the Department of Environmental Affairs and Tourism, and with the approval of the Prince Edward Islands Management Committee. Marion Island is a sub-Antarctic island (290 km^2) which is situated at 46°54'S and 37°45'E, and occurs approximately 2180km southeast of Cape Town, South Africa (Pistorius *et al*. 2001). The mice were sampled from seven localities across Marion Island (Fig. 1 Chapter 2). A total of 121 mice were caught using Sherman traps, baited with peanut butter and oats.

DNA extraction, amplification and sequencing

Both kidneys of each mouse were dissected out, placed in individual 1.5 ml eppendorf tubes on ice and transferred to a -20°C freezer for storage. DNA was extracted from the kidneys using the Roche high Pure PCR template preparation kit (Roche Applied Science) following the manufacturer's prescribed protocol for nucleic acid extraction from mammalian tissue.

Bacterial genome amplification was achieved using three primer sets (Table 1) targeting a region of ~900 bp, for *Streptococcus* and *Lactobacillus*, and a ~1.3 kb of the

16S rRNA gene using a universal primer set (Hauben *et al.* 1997), at reaction conditions and thermal cycle profiles specific for each primer set used. Amplification of the 16S gene target was undertaken to confirm bacterial genome presence and to obtain broad prevalence estimates. All kidney samples were subsequently screened with genus-specific primers, to obtain estimates of *Lactobacillus* and *Streptococcus* genus prevalence and to identify the proportion of bacterial co-infection of these genera within the samples.

Genomic amplification reactions were analysed for the presence of amplicons of the correct size by 1.5% agarose gel electrophoresis of 5µl of the product, against a 1kb size standard (GeneRuler, Fermentas). Negative and positive controls were included to preclude false positives due to reagent contamination, and false negatives, respectively. All positive PCRs were purified from the tube, with the exception of a few samples screened with the universal primers that resulted in multiple bands. These were gel-sliced purified, using a Roche PCR product purification kit (Roche, Mannheim Germany) according to manufacturer's specifications.

Cycle-sequencing reactions were performed at primer-specific annealing temperatures using Big Dye Terminator Cycle Sequencing Kit version 3.1 (Perkin-Elmer, Foster City, USA), with each of the external PCR primers (Table 1), and run on an AB3130. Sequence chromatograms were viewed, edited and aligned in the Chromas programme included in MEGA5 (Tamura *et al.* 2011). The resulting contig produced from the forward and reverse sequences was then blasted against the Genbank database (www.ncbi.nlm.nih.gov/blast) to identify the bacterial taxon present. The results of the BLAST searches were valuable for confirming the bacterial genus / species present in each sample and provided a preliminary indication of the bacterial species heterogeneity in the host *Mus musculus domesticus*.

Table 1 The 16S rRNA primer sets used in this study to obtain *Streptococcus*, *Lactobacillus* and overall bacterial prevalence estimates.

Primer set	Tm	Purpose
StrepF & Lepto1338r	57°C	*Streptococcus* amplification and characterization
StrepF & LactR	52°C	*Lactobacillus* amplification
16S27f & 16S1522r	59°C	Identification of bacterial genome with cultured samples

Phylogenetic analyses

Sequences generated for the *Streptococcus* dataset were initially aligned to the reference sequences downloaded from the Genbank database (Appendix A) using the ClustalW programme incorporated in MEGA5 (Tamura *et al*. 2011), and adjusted manually to further improve the alignment. Basic sequence statistics including the proportion of variable and parsimony informative sites, as well as base composition and the transition:tranversion ratio (R), were estimated in MEGA5. The program used to determine the model of evolution that best fitted the *Streptococcus* dataset was JModelTest v0.1 (Posada 2008). The best-fit model was selected under the Akaike Information Criterion (AIC) and used for all phylogenetic inferences performed using the *Streptococcus* dataset.

Neighbour-joining (NJ) and minimum evolution (ME) trees were inferred using pairwise deletion as well as complete deletion of gaps and missing data. This was done to evaluate the effect of differential treatment of both gaps and missing data on the resultant phylogenies. As there was no difference in the trees produced, pairwise deletions were used to ensure that missing data/gaps in the *Streptococcus* dataset were not deleted across the entire dataset.

Bayesian Inference (BI) analyses, performed using MrBayes v3.1 (Ronquist *et al*. 2005) made use of AIC-selected best-fit model priors from JModelTest (Posada 2008). The Markov chain Monte Carlo (MCMC) process was started from random starting points with four chains being run simultaneously for 10,000,000 generations, using default heating and swap parameters. Trees and parameters were recorded every 100 generations and the first 20 % of trees retained were discarded as the 'burn-in'. Two

independent runs were performed to ensure that the results converged. Maximum likelihood (ML) analyses were performed in PhyML v2.4.4 (Guindon and Gascuel 2003), with nodal support being assessed with 5000 bootstrap replications.

To evaluate the influence of parameter complexity on tree topology, an uncorrected *p*-distance tree was compared to the ME, ML and BI trees. As no topological differences were noted in any the analyses performed, a ME tree summarising observed changes is presented for the *Streptococcus* dataset (Fig. 1), with bootstrap values and posterior probabilities obtained from the ML and BI analyses being transferred to the appropriate nodes.

Culturing

Culturing was undertaken to obtain pure colony isolation of all potential novel *Streptococcus* species through four independent culture attempts. The first culture attempt was directed at the samples found by PCR screening to contain a novel *Streptococcus* species, described by de Bruyn *et al.* (2008) and collected in 2007. Tissue fragments were streaked onto blood agar plates inoculated with 5% cattle blood and incubated at 37ºC for 48 hours. The first culture attempt assessed the efficiency of both oxygen- as well as a carbon dioxide enriched environment, for each tissue sample. As a carbon dioxide enriched environment yielded more colony growth it was used in all subsequent culturing attempts.

The second culturing attempt was directed at the other potential novel *Streptococcus* species which were identified during the execution of a BSc(Hons) research project (Eadie 2008) using the same set of samples reported in the de Bruyn *et al.* (2008) study. The individuals that were not successfully cultured in the first attempt were also included in this culturing attempt. Kidney fragments were kept in brain-heart infusion (BHI) broth at 37°C for 48-60 hours before being streaked onto blood agar plates inoculated with 5% cattle blood and incubated for 60 hours at 37°C. The third culturing attempt was directed at samples used in this study which were collected from Marion Island in 2009. Tissue fragments were streaked onto blood agar plates inoculated with 5% cattle blood and incubated at 37ºC for 120 hours.

The fourth and final culturing attempt was directed at all the *Streptococcus* positive samples collected in 2007 and in 2009. Duplicate samples were used, with the first samples being incubated in BHI broth at 37°C for 24 hrs and then streaked out onto Columbia blood agar (supplemented with 5% cattle blood) plates and incubated for 36 hours at 37°C; while the second batch of tissue samples were streaked directly onto the Columbia blood agar plates. The samples on Columbia agar plates were then incubated at 37°C for 36hrs.

Pure colonies for all the culture attempts were obtained as follows: a small fragment of tissue was streaked onto a blood agar plate (enriched with cattle blood), except for the fourth culture attempt where they were streaked onto Columbia blood agar, and incubated at 37°C for 24 hours in a carbon dioxide enriched environment. If no growth was observed the plates were then left for another 24hours. Once colonies were observed they were re-streaked onto new blood agar plates for renewed nourishment. If there was more than one colony present on a plate, they were streaked out onto individual plates and labelled alphabetically (A/B/C). These plates were then re-incubated at 37°C for 48 hrs. After 48 hrs if there was still more than one colony present on a plate then the colonies were re-streaked and labelled alpha-numerically (A1/A2/A3). It should be noted that all samples showed some form of haemolytic growth which is typical of most streptococci. Once pure colonies were obtained the DNA was extracted using a Roche high pure PCR template preparation kit. The contiguous sequence obtained from the forward and reverse primers was used to identify the bacterium to genus level, and when possible, species level (Appendix B).

Results

Phylogenetic analyses

The alignment of the 16S rRNA gene sequences resulted in an homologous dataset of 765 base pairs. The final dataset contained 77 taxa comprising 62 *Streptococcus* and four *Enterococcus* species. Of the 765 nucleotides characterised, 628 (82.1%) were conserved, 135 (17.6%) were variable, two contained gaps inserted for alignment purposes and 86 (11.2%) of the variable sites were parsimony informative.

Estimation of TrN+I+G model parameters in JModelTest recovered a gamma distribution shape parameter (G) of 0.3320 and proportion of invariant sites (I) of 0.6480. The average nucleotide composition estimated under this best-fit model was 27.2%, 19.2%, 29.2% and 24.4% for A, C, G and T, respectively.

All three methods of phylogenetic reconstruction (ME with 10,000 bootstrap support (BS), BI with posterior probabilities and ML with 5000 BS) recovered trees with similar topologies. Nodes that had high levels of support from BI (>90%) also had high levels of support for terminal nodes in the ME and ML analyses. The ME analyses recovered one tree with a SBL value of 0.84705245.

Streptococcus *prevalence*

Of the 121 *Mus musculus* kidney samples tested with the *Streptococcus*-specific primer set, only nine were positive for *Streptococcus*, corresponding to an overall prevalence of 7.4%. The grouping of $^{8}/_{9}$ (88.9%) *Mus musculus* samples with an uncultured *Streptococcus* species found the in de Bruyn *et al.* (2008) study was well supported with all of the analyses performed. The grouping of *Mus musculus* 54 with *S. australis* and *S. parasanguinis* was not well supported with any of the analyses performed.

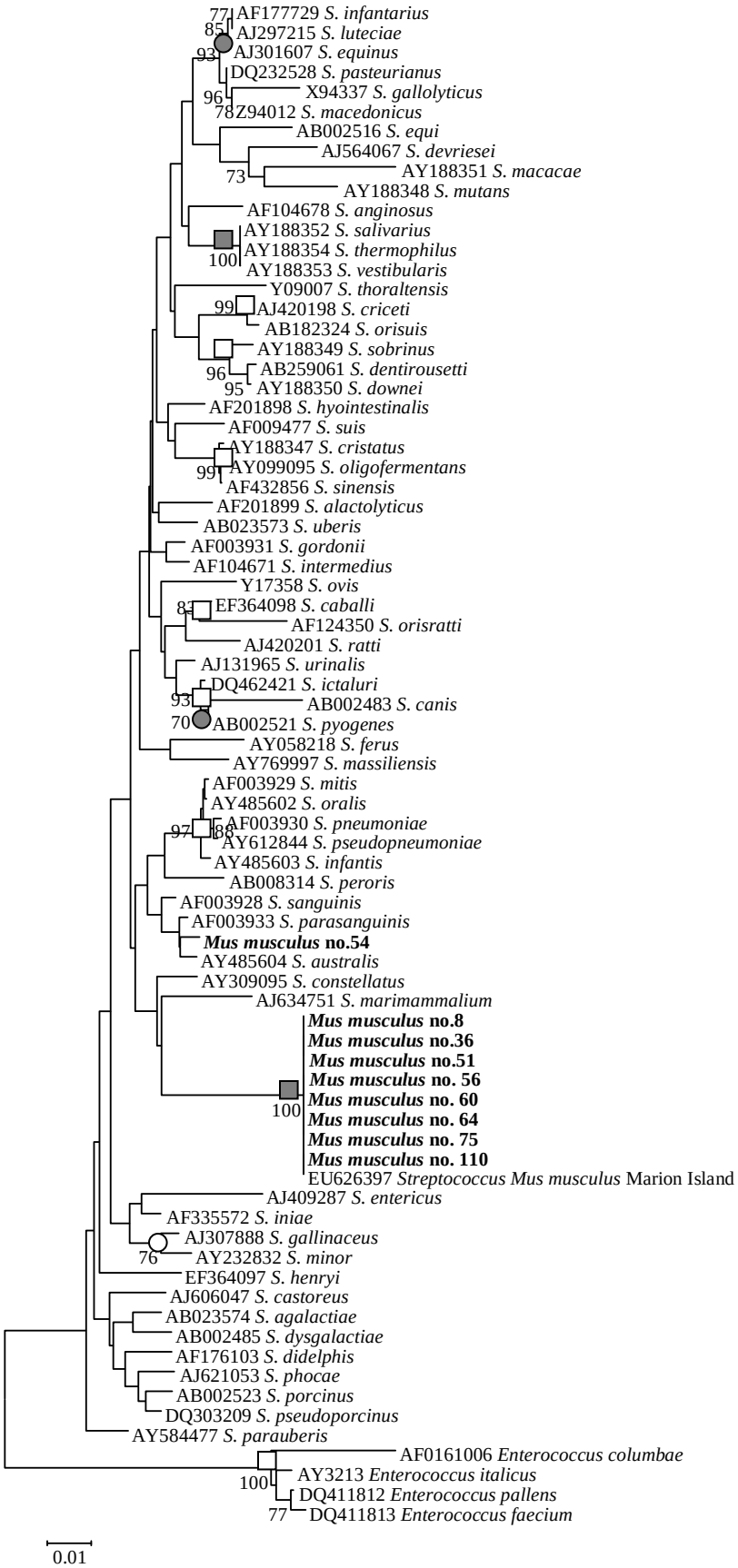
77
85
93
96
78
73
100
99
96
95
99
83
93
70
97
88
100
76
100
77
AF177729 S. infantarius
AJ297215 S. luteciae
AJ301607 S. equinus
DQ232528 S. pasteurianus
X94337 S. gallolyticus
Z94012 S. macedonicus
AB002516 S. equi
AJ564067 S. devriesei
AY188351 S. macacae
AY188348 S. mutans
AF104678 S. anginosus
AY188352 S. salivarius
AY188354 S. thermophilus
AY188353 S. vestibularis
Y09007 S. thoraltensis
AJ420198 S. criceti
AB182324 S. orisuis
AY188349 S. sobrinus
AB259061 S. dentirousetti
AY188350 S. downei
AF201898 S. hyointestinalis
AF009477 S. suis
AY188347 S. cristatus
AY099095 S. oligofermentans
AF432856 S. sinensis
AF201899 S. alactolyticus
AB023573 S. uberis
AF003931 S. gordonii
AF104671 S. intermedius
Y17358 S. ovis
EF364098 S. caballi
AF124350 S. orisratti
AJ420201 S. ratti
AJ131965 S. urinalis
DQ462421 S. ictaluri
AB002483 S. canis
AB002521 S. pyogenes
AY058218 S. ferus
AY769997 S. massiliensis
AF003929 S. mitis
AY485602 S. oralis
AF003930 S. pneumoniae
AY612844 S. pseudopneumoniae
AY485603 S. infantis
AB008314 S. peroris
AF003928 S. sanguinis
AF003933 S. parasanguinis
Mus musculus no.54
AY485604 S. australis
AY309095 S. constellatus
AJ634751 S. marimammalium
Mus musculus no.8
Mus musculus no.36
Mus musculus no.51
Mus musculus no. 56
Mus musculus no. 60
Mus musculus no. 64
Mus musculus no. 75
Mus musculus no. 110
EU626397 Streptococcus Mus musculus Marion Island
AJ409287 S. entericus
AF335572 S. iniae
AJ307888 S. gallinaceus
AY232832 S. minor
EF364097 S. henryi
AJ606047 S. castoreus
AB023574 S. agalactiae
AB002485 S. dysgalactiae
AF176103 S. didelphis
AJ621053 S. phocae
AB002523 S. porcinus
DQ303209 S. pseudoporcinus
AY584477 S. parauberis
AF0161006 Enterococcus columbae
AY3213 Enterococcus italicus
DQ411812 Enterococcus pallens
DQ411813 Enterococcus faecium
0.01

Fig. 1 A 50% majority rule consensus ME tree inferred in MEGA5 using pairwise deletion of gaps and missing data, the Tamura-Nei model of evolution and a gamma distribution shape parameter of 0.3. Bootstrap support values shown are those ≥ 70% obtained from 10,000 pseudoreplicates. Taxon names for reference sequences include the relevant Genbank accession numbers whilst samples characterised in this study are indicated in bold. Nodal support from the Bayesian and ML analyses are indicated as follows: open circles: 51-65 %, closed circles: 66-79 %, open squares: 80-95 % and closed squares: 96-100 %.

Culturing

In table 2 it is evident that the most predominant species cultured belonged to the genus *Staphylococcus*. For some individuals, more than one species was present indicating multiple bacterial co-inhabitants for that given sample. This was readily detected from attempts to sequence the *Streptococcus* (*Mus musculus* 10, 54 and 64) and 16S products amplified using the *Streptococcus* specific and universal primers respectfully, as mixed bacterial sequences were obtained following two or more sequencing attempts. *Mus musculus* 10 was excluded from the dataset as a homologous sequence was unattainable.

Table 2 Culturing and PCR screening results summary for the 121 kidney samples screened in this study.

Individual #	**Universal 16S screening**	***Streptococcus* screening Species recovered**	***Lactobacillus* screening**	**Species obtained from culturing**
1	–	–	–	
2	–	–	–	
3	–	–	–	
4	–	–	–	
5	–	–	–	
6	–	–	–	
7	–	–	–	
8	+	+ Novel *Streptococcus*	–	*Staphylococcus epidermis*
9	–	–	–	
10	+	–	–	*Micrococcus luteus*
11	–	–	+	
12	–	–	–	
13	–	–	–	
14	–	–	–	
15	–	–	–	

16	–	–		–		
17	–	–		+		
18	–	–		–		
19	–	–		–		
20	–	–		–		
21	–	–		–		
22	–	–		–		
23	–	–		–		
24	–	–		–		
25	–	–		–		
26	–	–		–		
27	–	–		–		
28	–	–		–		
29	+	–		–		
30	+	–		–		
31	+	–		–		
32	+	–		+		
33	–	–		–		
34	–	–		–		
35	+	–		–		
36	+	+	Novel *Streptococcus*	+	*Staphylococcus epidermis* & *Enterococcus faecalis*	
37	+	–		–		
38	+	–		+		
39	–	–		–		
40	–	–		–		
41	–	–		–		
42	–	–		–		
43	–	–		–		
44	+	–		–		
45	–	–		–		
46	–	–		–		
47	–	–		–		
48	–	–		–		
49	–	–		–		
50	–	–		–		
51	–	+	Novel *Streptococcus*	–	*Bacillus cereus*	
52	–	–		–		
53	–	–		–		
54	+	+	*S. parasanguinis*	–	*Staphylococcus epidermis*,	

					Enterococcus sp.
55	–	–		+	
56	+	+	Novel *Streptococcus*	–	*Staphylococcus saprophyticus*, *Staphylococcus equorum*
57	–	–		–	
58	–	–		–	
59	–	–		–	
60	+	+	Novel *Streptococcus*	–	
61	–	–		+	
62	–	–		–	
63	–	–		–	
64	–	+	Novel *Streptococcus*	–	
65	–	–		–	
66	–	–		–	
67	–	–		–	
68	–	–		–	
69	–	–		–	
70	–	–		–	
71	–	–		–	
72	–	–		–	
73	–	–		–	
74	–	–		–	
75	+	+	Novel *Streptococcus*	–	*Paenibacillus thiaminolyticus*
76	–	–		–	
77	–	–		+	
78	–	–		–	
79	–	–		–	
80	–	–		–	
81	–	–		–	
82	–	–		–	
83	–	–		–	
84	–	–		–	
85	–	–		+	
86	–	–		–	
87	–	–		–	
88	–	–		–	
89	–	–		–	
90	–	–		–	
91	–	–		–	

92	–	–		–
93	–	–		–
94	–	–		–
95	–	–		–
96	–	–		–
97	–	–		–
98	–	–		–
99	–	–		–
100	–	–		–
101	–	–		–
102	–	–		–
103	–	–		–
104	–	–		–
105	+	–		–
106	+	–		–
107	–	–		–
108	+	–		–
109	+	–		–
110	+	+	Novel *Streptococcus*	–
111	–	–		–
112	–	–		–
113	–	–		+
114	–	–		–
115	–	–		–
116	–	–		–
117	–	–		–
118	–	–		+
119	–	–		–
120	+	–		–
121	–	–		–

Discussion

Bacterial contamination of food and water via urine and faecal environmental contamination has been of concern to communities for more than a century (Hundesa *et al.* 2006). When invasive vertebrate species become established in new environments, such as *Mus musculus* on Marion Island, they may affect the native species through predation and competition, which has indirect effects on the native infectious diseases (Lafferty and Geber 2002). This is especially relevant when an invasive species can

potentially act as a reservoir host of diseases that native vertebrate species have had no previous exposure to (de Bruyn *et al*. 2008). However, it is important to consider that by virtue of their isolation or distance from neighbouring populations, island populations may harbor fewer pathogens than non-island populations (McCallum *et al*. 2001).

Although streptococci can constitute part of the normal flora of the human oral cavity, as well as the respiratory, genital and alimentary tracts (Rurangirwa *et al*. 2000; Vela *et al*. 2002; Glazunova *et al*. 2006), some streptococci also function as established and/or opportunistic pathogens (Collins *et al*. 2002). The taxonomy of the genus *Streptococcus* has been greatly improved as a result of the use of molecular chemical and genetic methods (Fernandez *et al*. 2004; Glazunonva *et al*. 2006), and 16S rRNA gene sequencing, in particular (Collins *et al*. 2002; Vela *et al*. 2002).

Streptococcus prevalence determined for *Mus* in this study was relatively low (7.4% overall) compared to the overall value of 30% reported previously (de Bruyn *et al*. 2008). The highest prevalence occurred at the Mixed Pickle site (55.6%), with Base, Kildalkey, Swartkops and Watertunnel each having a prevalence of 11.1%. This is suggestive of geographical clustering of cases to the western part of the island and indicates a possible host population infection rate difference in Marion Island mice, with respect to this bacterial genus. The majority of *Streptococcus* species were found in females (77.8%) with only two males (22.2%) being infected. The low overall prevalence in males is possibly due to the fact that males infected died before sampling, possibly due to testosterone influenced susceptibility to *Streptococcus* infection (Willoughby and Watson 1964; Medina *et al*. 2001).

The majority (88.9%) of the *Streptococcus* sequences obtained from the 2009 *Mus musculus* samples screened in this study, grouped with the novel *Streptococcus* species identified in the de Bruyn *et al*. (2008) study. As this bacterial lineage could not be cultured or biochemically characterised, its potential disease capabilities remain unknown. The *Mus musculus* (54) sample grouped most closely to *S. parasanguinis*, which is a species found in throats of children with no underlying disease as well as in patients with Kawasaki disease (Ohkuni *et al*. 1993). The remaining *Mus musculus* samples grouped most closely to *S. marimammalium*, which has been isolated from common and grey seals (Köhler 2007).

Using the BLAST program (www.ncbi.nlm.nih.gov/blast) the 16S rRNA gene analysis revealed that $^{8}/_{9}$ isolates were identical to the uncultured *Streptococcus* species found in the de Bruyn *et al*. 2008 study with closest sequence similarity to three previously described species, *S. constellatus*, *S. intermedius* and *S. anginsus* being 96%. The *Mus musculus* individual (54) was found to have 99% sequence similarity to *S. parasanguinis* when using the BLAST program. Even though it is not possible to delineate species on the basis of 16S rRNA sequences similarities, stains exhibiting 3-5% or greater sequence divergence are generally regarded as separate species (Collins *et al*. 2004).

Traditionally identification of *Streptococcus* species has been based on haemolytic activity and Lancefield grouping (Täpp *et al*. 2003). However, Lancefield groups are not species-specific (Farrow and Collins 1984; Lawrence *et al*. 1985) and haemolytic activity differs within species (α-, β- or γ haemolysis) and depends on incubation procedures, as well as origin of blood in the substrate (Täpp *et al*. 2003). This is evident in Appendix C as a variety of substrates and culturing conditions were used to grow the various *Streptococcus* species. Since the discovery of PCR and DNA sequencing, bacterial species gene sequence comparisons showed that the 16S rRNA gene is highly conserved within and among species of the same genus and can be used as the new standard for delineating bacterial species (Woo *et al.* 2002). However, Nikkari *et al*. (2002) urge that multiple broad-range primers must be used to detect polymorphisms in conserved primer recognition sites for unrecognized organisms when undertaking bacterial screening.

Although four culture attempts were undertaken, it was not possible to culture the novel *Streptococcus* species identified molecularly in this, and a previous study (de Bruyn *et al.* 2008). From Appendix C it is evident that in order to successfully grow novel species a wider variety of media and environmental conditions will have to be assessed in future studies. Identifying new *Streptococcus* species is fraught with difficulties as these are slow growing bacteria, requiring up to seven days to propagate, and their unequivocal identification is reliant on a classification system that does not always correlate with phylogenetic analysis (Facklam 2002; Picard *et al*. 2004).

With regards to environmental shedding and potential contamination, important factors to consider for a pathogen to maintain infectivity outside a host are temperature, relative humidity, desiccation and UV light (Sinclair *et al*. 2008). These factors are of particular importance to streptococci in that they are non-sporulating (Lawson *et al*. 2005a) and prone to desiccation. They are therefore unlikely to survive in the environment for long periods of time. However, this does not mean that the mice do not pose a threat to Marion Island vertebrates. The isolation of a novel *Streptococcus* species in *Mus* kidneys confirms the potential for disease transmission and excretion via urine to other species. The mice are found abundantly on beaches where they are attracted to the seals and seabirds faeces and carcasses (Avenant and Smith 2003). This interaction allows for the environmental contamination that could result in disease transmission to the seals and seabirds, which is of particular concern on the Western side of the Island where *Streptococcus* prevalence was highest.

Despite the substantial increase in newly described streptococcal species, it is evident from this study as well as others, that many streptococcal species remain to be discovered (Collins *et al*. 2002). Conservation biologists will begin to understand and minimize disease risks by monitoring for disease, reducing over-crowding, inbreeding and selection for susceptibility (Lafferty and Geber 2002).

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Appendix A. Summary of the *Streptococcus* and *Enterococcus* reference species used in this study.

Species	Genbank accession number	Reference
S. alactolyticus	AF201899	Unpublished
S. agalactiae	AB023574	Unpublished
S. anginosus	AF104678	Jacobs *et al.* 2000
S. australis	AY485604	Arbique *et al.* 2004
S. caballi	EF364098	Milinovich *et al.* 2008
S. canis	AB002483	Unpublished
S. castoreus	AJ606047	Lawson *et al.* 2005b
S. constellatus	AY309095	Takao *et al.* 2004
S. criceti	AJ420198	Whatmore & Whiley 2002
S. cristatus	AY188347	Unpublished
S. dentirousetti	AB259061	Takada & Hirasawa 2008
S. devriesei	AJ564067	Collins *et al.* 2004
S. didelphis	AF176103	Rurangirwa *et al.* 2000
S. downei	AY188350	Unpublished
S. dysgalactiae	AB002485	Unpublished
S. entericus	AJ409287	Vela *et al* 2002
S. equi	AB002516	Unpublished
S. equines	AJ301607	Unpublished
S. ferus	AY058218	Baele *et al.* 2003
S. gallinaceus	AJ307888	Collins *et al.* 2002
S. gordonii	AF003931	Unpublished
S. henryi	EF364097	Milinovich *et al.* 2008
S. hyointestinalis	AF201898	Unpublished
S. ictaluri	DQ462421	Shewmaker *et al.* 2007
S. infantis	AY485603	Arbique *et al.* 2004
S. infantarius	AF177729	Schlegel *et al.* 2000
S. iniae	AF335572	Bachrach *et al.* 2001
S. intermedius	AF104671	Jacobs *et al.* 2000
S. luteciae	AJ297215	Unpublished
S. macacae	AY188351	Unpublished
S. macedonicus	Z94012	Snaidr *et al.* 1997
S. marimammalium	AJ634751	Lawson *et al.* 2005a
S. massiliensis	AY769997	Glazunova *et al.* 2006
S. minor	AY232832	Vancanneyt *et al.* 2004
S. mitis	AF003929	Unpublished
S. mutans	AY188348	Unpublished
S. oligofermentans	AY099095	Tong *et al.* 2003
S. oralis	AY485602	Arbique *et al.* 2004
S. orisratti	AF124350	Zhu *et al.* 2000
S. orisuis	AB182324	Takada and Hirasawa 2007
S. ovis	Y17358	Collins *et al.* 2001
S. parasanguinis	AF003933	Unpublished
S. parauberis	AY584477	Picard *et al.* 2004
S. pasteurianus	DQ232528	Unpublished
S. peroris	AB008314	Kawamura *et al.* 1998
S. phocae	AJ621053	Gibello *et al.* 2005
S. pneumoniae	AF003930	Unpublished
S. porcinus	AB002523	Unpublished
S. pseudopneumoniae	AY612844	Arbique *et al.* 2004
S. pseudoporcinus	DQ303209	Bekal *et al.* 2006
S. pyogenes	AB002521	Unpublished

S. ratti	AJ420201	Whatmore and Whiley 2002
S. salivarius	AY188352	Unpublished
S. sanguinis	AF003928	Unpublished
S. sobrinus	AY188349	Unpublished
S. suis	AF009477	Chatellier *et al.* 1998
S. sinensis	AF432856	Woo *et al.* 2002
S. thermophilus	AY188354	Unpublished
S. thoraltensis	Y09007	Devriese *et al.* 1997
S. uberis	AB023573	Unpublished
S. urinalis	AJ131965	Collins *et al.* 2000
S. vestibularis	AY188353	Unpublished
E. columbae	NR041708	Patel *et al.* 1998
E. italicus	AY321375	Carvalho *et al.* 2004
E. pallens	DQ411812	Carvalho *et al.* 2008
E. faecium	DQ411813	Carvalho *et al.* 2008

Appendix B. Summary of the four cultures attempts undertaken in this study for samples collected in 2007 and 2009.

Individual #	**Year sampled**	**Genus/Species obtained Culture attempt 1**	**Genus/Species obtained Culture attempt 2**	**Genus/Species obtained Culture attempt 3**	**Genus/Species obtained Culture attempt 4**
2	2007	Exigobacterium, Curtobacterium & *Staphylococcus saprophytics*			Mixture
3	2007				*Micrococcus yunnanensis,* & *Staphylococcus epidermis*
7	2007				*Enterococcus faecalis* & *Staphylococcus epidermis*
8	2009			*Staphylococcus epidermis*	
10	2009				*Micrococcus luteus*
14	2007	*Staphylococcus sp.*			
36	2009				*Staphylococcus epidermis* & *Enterococcus faecalis*
41	2007	Uncult. *Neisseria, Streptococcus salivarius* & *Rothia mucilaginosa*			*Paenibacillus lautus*
43	2007	*Staphylococcus aureus*			
45	2007		*Enterococcus faecalis*		*Enterococcus faecalis*
49	2007		*Staphylococcus epidermis*		
51	2009				*Bacillus cereus*
54	2009			*Enterococcus* sp.	*Staphylococcus epidermis*
64	2009			*Staphylococcus equorum*	*Staphylococcus saprophyticus*
75	2009				*Paenibacillus thiaminolyticus*

Appendix C. Culturing conditions for the isolation of various *Streptococcus* species.

***Streptococcus* species**	**Host**	**Culture media**	**Duration of incubation (hrs)**	**Temp. (˚C)**	**Atmospheric environment**	**Reference**
S. iniae	Rainbow trout	Columbia agar with 5% sheep blood	24-48	24	NS	Bachrach *et al*. 2001
S. agalactiae	Bottlenose dolphin	Tryptic soy broth with sheep blood 5% sheep blood agar	3-4 18-24	27 27	NS	Evans *et al*. 2006
S. phocae	Salmon	Columbia blood agar	24-48	30	Aerobic	Gibello *et al*. 2005
S. massiliensis	Human	Sheep blood agar	48	37	5% CO_2 enriched	Glazunova *et al*. 2006
S. peroris and *S. infantis*	Human	Columbia blood agar with 5% sheep blood	NS	37	Aerobic	Kawamura *et al*. 1998
S. halichoeri	Grey seals	Columbia blood agar with 5% sheep blood	NS	37	Aerobic	Lawson *et al*. 2004
S. marimmalium	Common seal	Columbia agar with 5% sheep blood	NS	37	Capnophilic	Lawson *et al*. 2005a
S. castoreus	Beaver	Columbia blood agar with 5% horse blood	NS	37	Anerobic	Lawson *et al*. 2005b
S. phocae	Salmon	Columbia sheep blood agar Tryptone soy agar with 1% NaCl	48 48	22-25 22-25	Aerobic Aerobic	Romalde *et al*. 2007
S. didelphis	Opossum	Columbia agar with 5% sheep blood and MacConkey agar enriched with thioglycoll-ate broth	NS	37	NS	Rurangirwa *et al*. 2000
S. orisuis	Pig	Brain-heart infusion agar with 5% horse blood	NS	37	Anaerobic	Takada *et al*. 2007
S. dentirousetti	Bats	Brain-heart infusion agar with 5% horse blood	NS	37	Anaerobic	Takada *et al*. 2008
S. fryi	Dog	Blood agar with 5% sheep blood	NS	37	5% CO_2	Tomida *et al*. 2011
S. oligofermentans	Humans	Brain-heart infusion agar with 5% sheep blood	NS	37	N_2/CO_2 (95/5%)	Tong *et al*. 2003
S. minor	Dog	Columbia agar with 5% horse blood	24	37	Micro-aerobic	Vancanneyt *et al*. 2004
S. ferus	Rats	Brain-heart infusion broth with 0.5% (w/v) glucose	NS	37	Anaerobic	Whatmore and Wiley 2002
S. sinensis	Human	Sheep blood agar	24	37	Aerobic	Woo *et al*. 2002

S. orisratti	Rats	Columbia sheep blood agar	18	37	N_2/CO_2 (95/5%)	Zhu *et al*. 2000

*NS: Not stated in literature

CHAPTER 4

Helicobacter prevalence and diversity in house mice from Marion Island

Abstract

Helicobacter species have a wide host distribution but their pathogenic potential still remains largely unknown. The genus currently comprises of more than 30 species with new ones continuously being discovered. One hundred and twenty one *Mus musculus* specimens were sampled from seven geographically discrete localities across Marion Island to assess the reservoir potential of these invasive rodents. DNA was extracted from the gastrointestinal tracts (GIT) as well as the livers of these house mice (*Mus musculus domesticus*) to determine *Helicobacter* genome presence using a 16S rRNA lineage-specific PCR approach. An overall *Helicobacter* infection rate of 12.4% was recovered with 86.7% of PCR-positive samples occurring in the GIT and 13.3% in the livers. Of the five *Helicobacter* lineages identified in this study, two correspond to previously described species *viz. H. typhlonius* and *H. hepaticus*. The remaining three *Helicobacter* species appear to be novel and will need to be cultured and biochemically characterized.

Keywords: *Helicobacter*, bacterial prevalence, *Mus musculus*, Marion Island, novel species

Introduction

Helicobacter are microaerophilic, gram-negative, highly motile, spiral shaped bacteria (Baele *et al*. 2008a). They are classified within the phylum Proteobacteria, class Epsilonproteobacteria, order Campylobacterales and family Helicobacteraceae. The *Helicobacter* genus is characterized by two major evolutionary lineages with more than

30 species being described (Comunian *et al.* 2006). Of these, *H. pylori* and *H. felis* are the two best known *Helicobacter* pathogens infecting humans (Fritz *et al.* 2006). Possible routes of transmission for *H. pylori* include oral-oral, faecal-oral and person to person (Dube *et al.* 2009); however the most common way to acquire *H. pylori* is through the consumption of contaminated water (Engstrand 2001). This bacterium colonizes the gastric mucosa where it establishes a chronic infection that is associated with an inflammatory response (Engstrand 2001). Due to the widespread and persistent nature of *H. pylori*, a high proportion of humans are at risk of peptic ulcers and gastric tumours (Correa 1992; Solnick *et al*. 2006), added to which there is a positive correlation between prevalence and host age (Fiedorek *et al.* 1991).

A number of *Helicobacter* species are known to occur in rodents, and particularly in mice. The *Helicobacter* species most commonly found in mice are *H. bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rappini*, *H. rodentium* and *H. typhlonius* (Whary and Fox 2006). A few of these *Helicobacter* species are pathogenic to mice, namely *H. hepaticus* and *H. bilis*, causing chronic active hepatitis and/or inflammatory bowel disease (Shomer *et al*. 1997; Ward *et al.* 1996). The vast majority of infected animals display no clinical signs of infection (Whary and Fox 2006). As *Helicobacter* occur in the mucosa and are passed on and transmitted via the faecal-oral route, one of the tissue samples targeted for molecular analysis of *Helicobacter* prevalence in Marion Island *Mus*, will be the gastro-intestinal tract (GIT). Bacterial presence will also be evaluated in the liver as hepatic forms of *Helicobacter* are known to occur (Fox *et al*. 1994; Patterson *et al*. 2000).

Materials and methods

Sampling

The study site was sub-Antarctic Marion Island (290 km^2) which is situated at 46°54'S and 37°45'E, and occurs approximately 2180 km southeast of Cape Town, South Africa (Pistorius *et al*. 2001). The mice were collected from Marion Island with permission from the Department of Environmental Affairs and Tourism, and with the approval of the Prince Edward Islands Management Committee. The mice were sampled

from seven localities across Marion Island, namely Base, Cape Davis, Kildalkey, Mixed pickle, Repettos, Swartkops and Watertunnel (fig. 1 Chapter 2). A total of one hundred and twenty one mice were caught using Sherman traps, baited with peanut butter and oats.

DNA extraction, amplification and sequencing

A fragment of tissue from the gastro-intestinal tract (GIT) and liver of each mouse was dissected out, placed in individual 1.5 ml eppendorf tubes on ice and transferred to a -20°C freezer for storage. DNA was extracted from the GIT and livers using the Roche high Pure PCR template preparation kit (Roche Applied Science) following the manufacturer's prescribed protocol for nucleic acid extraction from mammalian tissue.

Bacterial genome amplification was achieved using genus-specific primers targeting a ~850bp fragment of the 16S gene, at reaction conditions and thermal cycle profiles specific for the primer set used (Mostert 2010). Amplification of the gene target was taken to confirm bacterial presence and was used to obtain prevalence estimates.

Amplified samples were analysed for the presence of amplicons of the correct size by 1.5% agarose gel electrophoresis of 5µl of the polymerase chain reaction (PCR) product, against a 1kb size standard (GeneRuler Fermentas) ladder. Negative and positive controls were included to preclude false positives due to reagent contamination, and false negatives, respectively. All positive PCRs were purified from the tube using a Roche PCR product purification kit (Roche, Mannheim Germany) according to manufacturer's specifications. Cycle-sequencing reactions were performed at primer-specific annealing temperatures using Big Dye Terminator Cycle Sequencing Kit version 3.1 (Perkin-Elmer, Foster City, USA), with each of the external PCR primers, and run on an AB3130. Sequence chromatograms were viewed, edited and aligned in the Chromas programme included in MEGA4 (Tamura *et al.* 2007). Each nucleotide sequence was then blasted against the Genbank database (www.ncbi.nlm.nih.gov/blast) to identify the bacterium present to genus level. This confirmed the target bacterial presence and provided a preliminary assessment of the bacterial species heterogeneity in the host *Mus musculus domesticus.*

Data analyses

Sequences generated were initially aligned to the reference sequences downloaded from the Genbank database (Table 1) using the ClustalW programme incorporated in MEGA4 (Tamura *et al*. 2007), and adjusted manually to further improve the alignment. Basic sequence statistics including the proportion of variable and parsimony informative sites, as well as base composition and the transition:tranversion ratio (R), were estimated in MEGA4. JModelTest v0.1 (Posada 2008) was used to determine the model of evolution that best fitted the dataset. The best-fit model was selected under the Akaike Information Criterion (AIC) and used for all phenetic analyses.

Table 1 *Helicobacter* reference species and their Genbank accession numbers used in this study.

Species	**Genbank accession number**	**Reference**
H. acinonychis	M88148	Unpublished
H. anseris	DQ415545	Fox *et al.* 2006
H. apodemus	AF284754	Unpublished
H. aurati	AF297868	Patterson *et al.* 2000
H. baculiformis	EF070342	Baele *et al.* 2008a
H. bilis	U18766	Fox *et al.* 1995
H. bizzozeronii	Y09404	Jalava *et al.* 1997
H. brantae	DQ415546	Fox *et al.* 2006
H. callitrichis	AY192526	Won *et al.* 2007
H. canadensis	AF262037	Fox *et al.* 2000
H. canis	AY631945	Dewhirst *et al.* 2005
H. cetorum	AF292378	Harper *et al.* 2000
H. cholecystus	AY686606	Dewhirst *et al.* 2005
H. cinaedi	M88150	Unpublished
H. cynogastricus	DQ004689	Van den Bulck *et al.* 2006
H. equorum	DQ307735	Moyaert *et al.* 2007
H. felis	M57398	Paster *et al.* 1991
H. fennelliae	M88154	Dewhirst *et al.* 2005
H. ganmani	AF000221	Robertson *et al.* 2001
H. hepaticus	U07575	Fox *et al.* 1994

H. kirbridei	AY554129	Unpublished
H. macacae	EF526074	Fox *et al.* 2007
H. marmotae	AF333340	Fox *et al.* 2001
H. mastomyrinus	AY742307	Shen *et al.* 2005
H. mesocricetorum	AF072471	Simmons *et al.* 2000
H. muricola	AF264783	Won *et al.* 2002
H. muridarum	M80205	Lee *et al.* 1992
H. mustelae	M35048	Paster *et al.* 1991
H. pametensis	M88147	Dewhirst *et al.* 1994
H. pullorum	AY631956	Dewhirst *et al.* 2005
H. pylori	U01330	Eckloff *et al.* 1994
H. rodentium	U96296	Shen *et al.* 1997
H. salomonis	U89351	Jalava *et al.* 1997
H. suis	EF204589	Baele *et al.* 2008b
H. suncus	AB006148	Goto *et al.* 1998
H. trogontum	U65103	Mendes *et al.* 1996
H. typhlonius	AF127912	Fox *et al.* 1999
H. winghamensis	AF363063	Melito *et al.* 2001
W. succinogenes	M88159	Wesley *et al.* 1995

Neighbour-joining (NJ) and minimum evolution (ME) trees were inferred using pairwise deletion as well as complete deletion of gaps and missing data. This was done to evaluate the effect of differential treatment of both gaps and missing data on the resultant phylogenies. As there was no difference in the trees produced, pairwise deletions were used to accommodate missing data/gaps in the datasets.

Maximum parsimony (MP) analyses were performed in PAUP* (Swofford 2003). The initial trees were obtained by performing a random heuristic search with 100 replications; while gaps were treated as a 5th character state. The number of trees retained from the heuristic search performed with tree bisection-reconstruction (TBR) branch swapping was seventeen and nodal support was assessed by using 1000 bootstrap replications. Bayesian Inference (BI) analyses, performed using MrBayes v3.1 (Ronquist *et al.* 2005) made use of AIC-selected best-fit model priors from JModelTest (Posada 2008). The Markov chain Monte Carlo (MCMC) process was started from random starting points with four chains being run simultaneously for 10,000,000 generations,

using default heating and swap parameters. Trees and parameters were recorded every 100 generations and the first 20 % of trees retained were discarded as the 'burn-in'. Two independent runs were performed to ensure that the results converged. Maximum likelihood (ML) analyses were performed in PhyML v2.4.4 (Guindon and Gascuel 2003), with nodal support being assessed with 5000 bootstrap replications.

To evaluate the influence of parameter complexity on tree topology, an uncorrected *p*-distance tree was compared to the MP, ML and BI trees. As no topological differences were noted in any the analyses performed, a ME tree summarising observed changes is presented for the dataset (Fig. 1), with bootstrap values and posterior probabilities obtained from the MP, ML and BI analyses being transferred to the appropriate nodes.

Statistical analyses

Statistical correlations between *Helicobacter* prevalence data and (1) mice sex and (2) sample localities where mice were caught, were tested for using General Linear Models in Statistica V9 (StatSoft Inc. 2010). Differences between infection prevalence by sex and sampling locality were investigated using multiple regression (Table 2).

Table 2 Infection prevalence of male and female *Mus musculus* mice across Marion Island localities, with the percentage of total individuals caught at each locality indicated in brackets.

	Kildalkey	**Repettos**	**Swartkops**	**Watertunnel**	**Mixed pickle**	**Cape Davis**	**Base**
Total infected	0 (0%)	3 (19%)	0 (0%)	2 (16.7%)	3 (10%)	6 (30%)	2 (10%)
Total not infected	18 (100%)	13 (81%)	5 (100%)	10 (83.3%)	27 (90%)	14 (70%)	18 (90%)
Number of males	8 (44%)	10 (62.5%)	4 (80%)	9 (75%)	15 (50%)	13 (65%)	15 (75%)
Number of females	10 (56%)	6 (37.5%)	1 (20%)	3 (25%)	15 (50%)	7 (35%)	5 (25%)
Number of males infected	0 (0%)	2 (12.5%)	0 (0%)	2 (16.6%)	0 (0%)	5 (25%)	2 (10%)
Number of females infected	0 (0%)	1 (6.5%)	0 (0%)	0 (0%)	3 (10%)	1 (5%)	0 (0%)

Results

Phylogenetic analyses

The alignment of the 16S rRNA gene sequences resulted in a homologous dataset of 769 base pairs. The final dataset contained 55 taxa which represented 39 *Helicobacter* species. The dataset comprised of 599 (77.9%) conserved, 165 (21.5%) variable, 123 (16.0%) parsimony informative and 42 (5.5%) singleton sites. Estimation of GTR+I+G model parameters in JModelTest recovered a gamma distribution shape parameter (G) of 0.5230 and proportion of invariant sites (I) of 0.6760. The average nucleotide composition estimated for this best-fit model selected under the AIC was 28.5%, 20.9%, 27.6% and 23% for A, C, G and T, respectively.

All four methods of phylogenetic reconstruction (ME with 100,000 bootstrap support (BS), MP with 1000 BS, BI with posterior probabilities and ML with 5000 BS)

recovered trees with similar topologies. Terminal nodes with high posterior probabilities (>90%) from Bayesian inference (BI) generally also had high (>70%) bootstrap support in the MP analyses. Nodes that had high levels of support from BI (>90%) and MP (>70%) also had high levels of support for terminal nodes in the ME and ML analyses. The ME analyses recovered one tree with a SBL value of 0.73601700. Although the MP and BI analyses recovered trees with similar topology, the terminal nodes had higher levels of resolution in the Bayesian tree. An un-weighted parsimony analysis recovered eleven trees of 559 steps with CI = 0.322, RI = 0.676, RCI = 0.218 and HI = 0.678; while re-weighting with CI resulted in one tree 190.23 in length, with CI = 0.435, RI = 0.754, RCI = 0.328 and HI = 0.565.

Helicobacter *prevalence*

Fifteen *Mus musculus* tested positive for *Helicobacter* corresponding to an overall prevalence of 12.4%. Of these, thirteen GI tract samples and three liver samples tested positive for *Helicobacter*, resulting in an overall prevalence of 10.7% and 2.5% for the GI tract and liver, respectively. One individual (*Mus musculus* 100) tested positive for *Helicobacter typhlonius* in both the GI tract and the liver.

The grouping of $^{7}/_{15}$ (46.7%) *Mus musculus* samples with *H. typhlonius* was well supported in all of the analyses performed. *H. hepaticus* and *H. cinaedi* each occurred in $^{3}/_{15}$ (20%) of the PCR-positive *Mus musculus* samples. Clustering of *Helicobacter* lineages from Marion Island *Mus*, with each of these reference strain sequences had high levels of support with all methods of inference. The grouping of *H. cetorum* and a lineage 2 positive *Helicobacter* strain identified in *Mus musculus* 52 was consistent throughout all the analyses; however, the node was not very well supported. *Mus musculus* 91 clustered with lineage two in all the analyses, except the NJ cluster analysis.

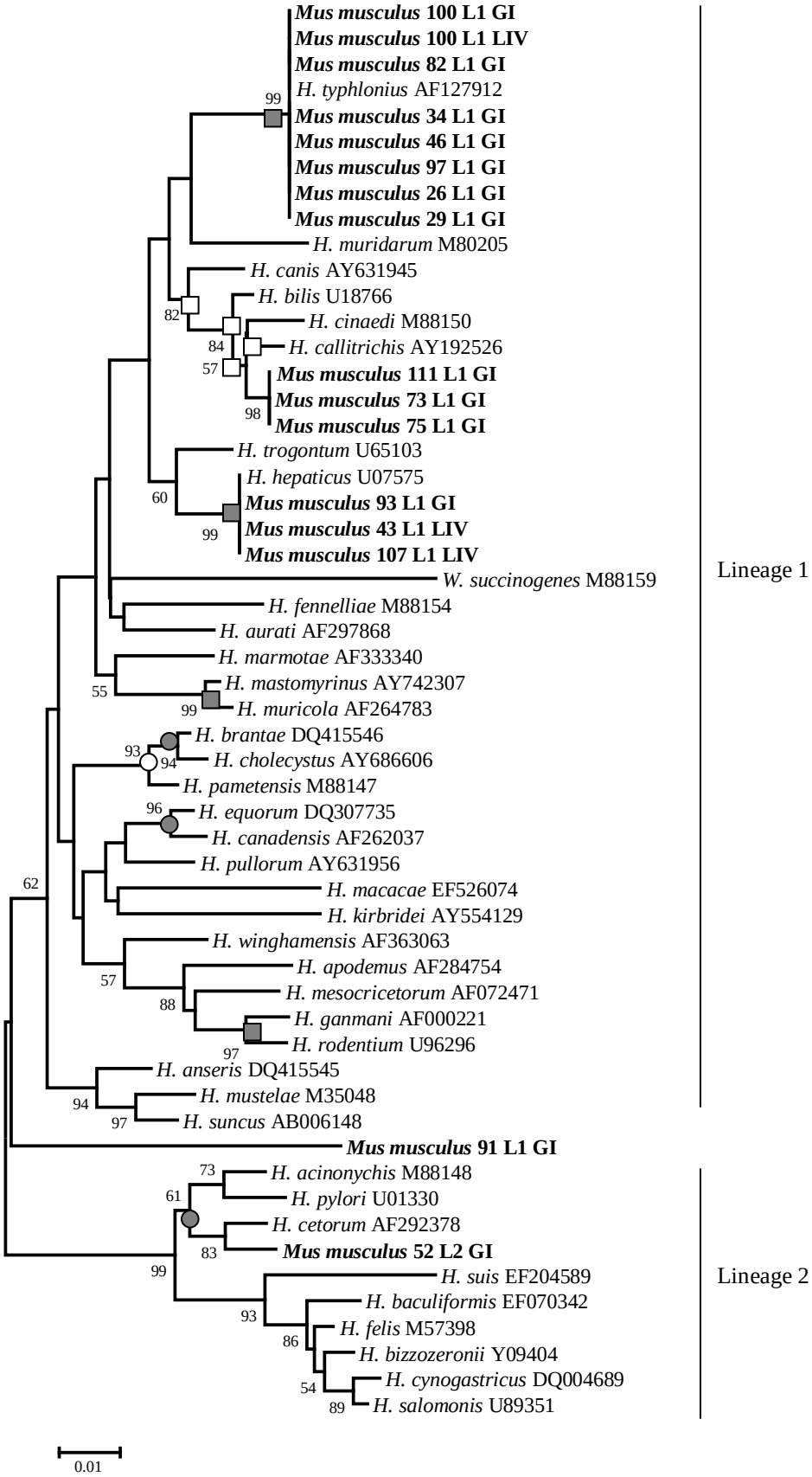
Mus musculus 100 L1 GI
Mus musculus 100 L1 LIV
Mus musculus 82 L1 GI
H. typhlonius AF127912
Mus musculus 34 L1 GI
Mus musculus 46 L1 GI
Mus musculus 97 L1 GI
Mus musculus 26 L1 GI
Mus musculus 29 L1 GI
H. muridarum M80205
H. canis AY631945
H. bilis U18766
H. cinaedi M88150
H. callitrichis AY192526
Mus musculus 111 L1 GI
Mus musculus 73 L1 GI
Mus musculus 75 L1 GI
H. trogontum U65103
H. hepaticus U07575
Mus musculus 93 L1 GI
Mus musculus 43 L1 LIV
Mus musculus 107 L1 LIV
W. succinogenes M88159
H. fennelliae M88154
H. aurati AF297868
H. marmotae AF333340
H. mastomyrinus AY742307
H. muricola AF264783
H. brantae DQ415546
H. cholecystus AY686606
H. pametensis M88147
H. equorum DQ307735
H. canadensis AF262037
H. pullorum AY631956
H. macacae EF526074
H. kirbridei AY554129
H. winghamensis AF363063
H. apodemus AF284754
H. mesocricetorum AF072471
H. ganmani AF000221
H. rodentium U96296
H. anseris DQ415545
H. mustelae M35048
H. suncus AB006148
Mus musculus 91 L1 GI
H. acinonychis M88148
H. pylori U01330
H. cetorum AF292378
Mus musculus 52 L2 GI
H. suis EF204589
H. baculiformis EF070342
H. felis M57398
H. bizzozeronii Y09404
H. cynogastricus DQ004689
H. salomonis U89351
Lineage 1
Lineage 2
0.01

Fig. 1 A 50% majority rule consensus ME tree inferred in MEGA4 using pairwise deletion of gaps and missing data and the Tamura-Nei nucleotide model of evolution with a gamma shape distribution parameter of 0.5230. Bootstrap support values shown are those ≥ 50% obtained from 100000 pseudoreplications. Taxon names for reference sequences include the relevant Genbank accession numbers. Samples used in this study are indicated in bold, with gastrointestinal (GI) and liver (LIV) indicated next to the sample names. Node support for parsimony, Bayesian and ML are indicated as follows: open circles: 51-65 %, closed circles: 66-79 %, open squares: 80-95 % and closed squares: 96-100 %.

Statistical analyses

Multiple regression analyses showed no correlation between *Helicobacter* infection prevalence with respect to sex (F = 0.52; d.f. = 1; n = 2; P = 0.47) or sampling locality (F = 0.89; d.f. = 1; n = 2; P = 0.35). This may be attributed to the low prevalence of *Helicobacter* (10.7% in the GI tract and 2.5% in the liver) as well as the small sample size when samples are partitioned by sampling locality.

Discussion

Rodents may be a source of *Helicobacter* infection that could be directly or indirectly acquired by humans; however this is not explored much in the literature (Comunian *et al.* 2006). The tropism of the *Helicobacter* genus includes the stomach, cecum, colon, liver and genital tract of mammals and birds (Dewhirst *et al.* 2005). The murine *Helicobacters* tend to colonize extragastric sites, preferentially the large intestine and the liver (Patterson *et al.* 2000), with the enterohepatic *Helicobacter* species being exploited to better understand Inflammatory Bowel Disease (IBD) in genetically susceptible mice (Solnick *et al.* 2006). The *Helicobacter* genus has a wide host distribution and pathogenic potential and it is clear that *Helicobacter* species can infect several animal species as well as colonise different anatomical regions of the gastrointestinal system (Fox *et al.* 1994).

The intestine of humans and animals are colonized with a complex microflora of indigenous aerobic and anaerobic bacteria, making the identification of a single pathogen difficult (Cahill *et al.* 1997). The analysis of the 16S rRNA gene region has become the primary method for determining prokaryotic phylogeny as it is useful in defining

relationships from phylum to species (Dewhirst *et al.* 2005). However, bacteria that seemingly belong to the same species on the basis of the 16S rRNA gene sequences can nevertheless be identified as different species on the basis of DNA/DNA hybridization and phenotypic analyses (Fox *et al*. 1992). Dewhirst *et al*. (2005) recently suggested that the rapidly changing 23S rRNA gene is more reliable than 16S rRNA for identification, classification and reflection of phylogenetic relationships within the genus *Helicobacter* due to the threefold higher number of informative bases. Nevertheless, it is increasingly evident that PCR methods for *Helicobacter* detection are preferred as they have been extensively described and used owing to the cost effectiveness, as well as the high sensitivity and specificity for identifying rodent *Helicobacters* to the genus or species level (Whary and Fox 2006).

Several species of *Helicobacter* infecting rodents have been described, including *H. hepaticus*, *H. bilis* and *H. rodentium*, which have been shown to colonise the liver and the intestine of immuno-deficient mice (Ward *et al*. 1996; Shomer *et al*. 1998; Maggio-Price *et al.* 2002; Shen *et al*. 2005). *H. hepaticus* was first identified in a long-term toxicology study in which the control group was found to have an extremely high incidence of hepatic tumors (Fox *et al.* 1994). *H. hepaticus* colonises the lower bowel of mice, which then shed the bacterium in their faeces promoting horizontal transmission through the faecal-oral route (Whary and Fox 2006). Currently, most commercial facilities screen their mouse colonies on a routine basis to establish that they are free of *H. hepaticus*, since that bacterium was found to cause chronic hepatitis and hepatic tumors (Patterson *et al*. 2000). In addition, there is some evidence that intestinal *Helicobacters* such as *H. cinaedi* and *H. fennelliae* may play a primary role in the development of human IBD (Totten *et al*. 1985).

Helicobacter prevalence was relatively low (12.4% overall), with the highest prevalence occurring at the Cape Davis (37.5%), Repettos (18.8%) and Mixed Pickle (18.8%) sampling sites. This is suggestive of geographical clustering of cases to the northern part of the island and indicates a possible host population infection rate difference in Marion Island mice, with respect to this bacterial genus. The majority of *Helicobacter* species were found in males (66.7%) with only five females (33.3%) being infected. A higher *Helicobacter* prevalence may have been observed if we looked at

colonic mucosa, as Comunian *et al*. (2006) found a higher prevalence in the colonic mucosa (30.7%) than the gastric mucosa (1.3%) of wild rodents in Brazil.

A large proportion of the *Mus musculus* samples grouped with 99% sequence identity to *H. typhlonius*; which is known to occur in mice and to cause IBD (Fox *et al.* 1999). To make sure that these samples are indeed *H. typhlonius* a second gene region should be included for future studies, as a 99% sequence similarity for *Helicobacter* does not always guarantee species identity (Fox *et al.* 1992; Dewhirst *et al.* 2005). The *Mus musculus* sample that grouped most closely to *H. cetorum* is unusual as *H. cetorum* is a species that was first isolated from the gastric mucosa of dolphins. When taking genetic distance into account it is evident that the three lineages represented by (i) *Mus musculus* 52, (ii) *Mus musculus* 91 and the (iii) samples 73, 75 and 111 each represent a new species. The genetic distance between *H. cinaedi* and *Mus musculus* 73, 75 and 111 is 1.33%; which is double that between the formally recognized species *H. muricola* and *H. mastomyrinus* and between *H. brantae* and *H. cholecystus* which have genetic distances of 0.66% and 0.67%, respectively. Given the rate at which new *Helicobacter* species have been described in recent years and the large number of *Helicobacter* spp. found in mice it is not surprising that three novel species have been identified in mice from Marion Island.

It is important to keep in mind that co-infection of *Helicobacter* species is not uncommon (Fritz *et al.* 2006); however this was not the case in this study. Genetic and socio-economic factors can also contribute to the acquisition of *Helicobacter* infection, with developing countries having higher prevalences than developed countries (Correa 1992; Engstrand 2001), owing to the hygiene standards, over-crowding and the contamination of water sources (Dube *et al.* 2009). This is evident for *H. pylori* prevalence and rate of infection, which is inversely related to the standard of living and sanitary practice in developing countries (Dube *et al.* 2009). The observation that mice may be genetically susceptible to disease depending on their *Helicobacter* infection status, places emphasis on determining *Helicobacter* status for all mouse experiments (Solnick *et al.* 2006).

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CHAPTER 5

Bartonella prevalence in invasive *Mus musculus domesticus*

Abstract

There is an increasing number of natural animal reservoirs particularly rodents, that are responsible for disease manifestation in humans. More specifically, there are 19 formally recognised *Bartonella* species, of which many have shown zoonotic potential with *Bartonella bacilliformis* and *Bartonella quintana* being the only two human-specific pathogens within the genus. Although a wide range of endemic and invasive commensal murid rodent species have been assessed for *Bartonella* prevalence, the domestic house mouse, *Mus musculus* remains one of the most under-studied commensal species. *Bartonella* prevalence has not been evaluated in *M. m. domesticus* from sub-Antarctic Marion Island, an invasive species which was introduced to this island by sealers in the 1800s. As rodent numbers have steadily increased since the removal of feral cats and as contact opportunities between mice and native vertebrates as well as researchers based at the island have increased in recent years, we set out to determine the prevalence and diversity of this bacterial genus which contains a number of species with zoonotic potential. To this end 171 *Mus musculus* individuals were sampled from throughout Marion Island and evaluated for *Bartonella* genome presence by amplification of the citrate synthase (*gltA*) gene. Negative and positive controls were included to preclude the possibility of false positive and false negative results, respectively. All samples screened were negative, corresponding to an overall prevalence of 0%. The results contrast markedly with the generally high levels of prevalence reported for other murid rodent species, and indicate that, possibly due to a founder effect, *Mus musculus* are not reservoirs of *Bartonella* infection on Marion Island.

Keywords: *Bartonella*; zoonotic potential; Marion Island; *Mus musculus*; citrate synthase gene

Introduction

Bartonella are fastidious, aerobic, gram-negative bacteria that can be found as bacilli or coccobacilli in the red blood cells of vertebrate hosts (Breitschwerdt and Kordick 2000). They are classified within the phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales and family Bartonellaceae. The *Bartonella* genus is currently composed of 19 species (Gundi *et al.* 2009), of which almost half have known zoonotic potential. The genus consists of two human-specific pathogens, *Bartonella bacilliformis* and *Bartonella quintana*, which cause Carrion's disease and trench fever, respectively (Berglund *et al*. 2010). Infection with rodent *Bartonella* spp. such as *B. elizabethae* and *B. vinsonii* subsp. *arupensis* in humans can result in endocarditis and febrile illness, respectively (Daly *et al.* 1993, Welch *et al.* 1999). *Bartonella* species recognised as causative agents of culture-negative and afebrile infective endocarditis may also be associated with prolonged bacteremia (Raoult *et al*. 1996).

Genetic susceptibility, age-associated immunocompetence, and pregnancy are factors that can influence *Bartonella* infection (Breitschwerdt and Kordick 2000). Congenital transmission could also contribute to a predilection of a given *Bartonella* species for infecting a specific host species more frequently (Breitschwerdt and Kordick 2000). The majority of human infections in North America and Europe have been attributed to two species, *B. henselae* and *B. quintana* (Houpikian and Raoult 2001). In South Africa, a prevalence of 10% for *B. henselae*, was recovered in a study on HIV-positive patients, with only one person of the one hundred and eighty eight screened showing clinical signs of infection (Frean *et al.* 2002).

Bartonella are transmitted by a variety of haematophagous invertebrates including ticks (Welch *et al.* 1999), fleas (Chomel *et al*. 1996) and lice (Roux and Raoult 1999). All *Bartonella* species are believed to be vector-transmitted, blood-borne, intracellular organisms with transmission being influenced by vector-host preferences (Breitschwerdt and Kordick 2000). Rodents generally have high levels of infection (Pretorius *et al.* 2004; Bastos 2007), and if occurring in close contact with humans represent a reservoir of

infection (Breitschwerdt and Kordick 2000). Simultaneous infection with more than one *Bartonella* species has also been documented in rodents (Kosoy *et al.* 1997).

The bacteria can be cultured from blood (Gundi *et al*. 2009), endothelial cells of lymph nodes (Wesslen *et al*. 2001), spleen (Infante *et al.* 2008) and liver (Gundi *et al.* 2010). However, as it takes up to six weeks to culture this fastidious bacterium, bacterial detection is more often reliant on molecular-based methods for diagnosis as they are highly sensitive and specific (Agan and Dolan 2002). As *Bartonella* is an erythrocyte-infecting bacterium (Berglund *et al*. 2010), blood-rich organs such as the heart and liver are suitable for molecular screening of *Bartonella* genome presence.

Material and methods

Sampling

The study site was the sub-Antarctic Marion Island (290 km^2) which is situated at 46°54'S and 37°45'E, and occurs approximately 2180 km southeast of Cape Town, South Africa (Pistorius *et al*. 2001). The mice were collected from Marion Island with permission from the Department of Environmental Affairs and Tourism, and with the approval of the Prince Edward Islands Management Committee. The mice were sampled from seven sites across Marion Island in 2009 and from four sites in 2007 (Fig. 1).

Genomic extraction and amplification

Kabeya *et al.* (2003) determined that the most successful sample material used to detect *Bartonella*, whether for culturing or polymerase chain reaction (PCR) analysis, was the liver. Thus, DNA was extracted from the liver samples using the Roche high Pure PCR template preparation kit (Roche Applied Science) following the manufacturer prescribed protocol for nucleic acid extraction from mammalian tissue. A 513 bp fragment of the citrate synthase gene (*gltA*) was targeted with genus-specific primers, at reaction conditions and thermal cycle profiles specific for the primer set used. Amplification of the gene target was taken to confirm bacterial presence and was then used to obtain prevalence estimates.

Samples were evaluated for the presence of amplicons of the correct size by 1.5 % agarose gel electrophoresis of 5 µl of the PCR product, against a 1 kb size standard (GeneRuler, Fermentas) ladder. Negative and positive controls were included to preclude false positives due to reagent contamination, and false negatives, respectively.

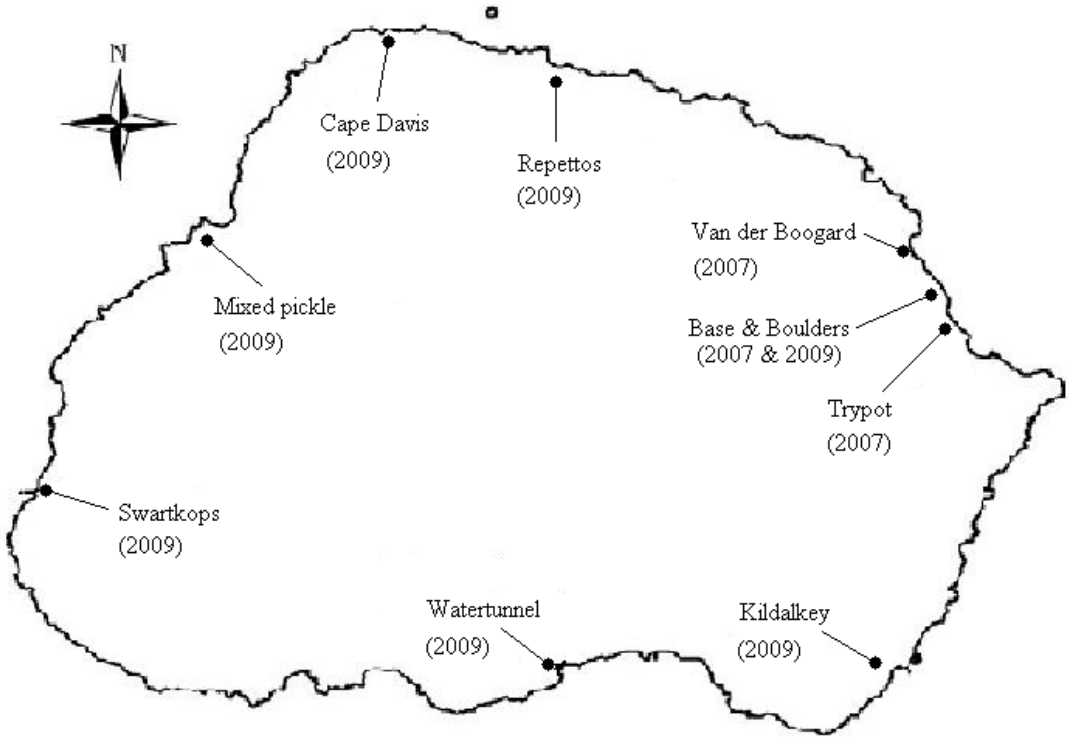

Fig. 1 Sampling localities of mice captured during 2007 and 2009.

Results

No evidence of *Bartonella* genome presence was found in the liver samples with the citrate synthase (*gltA*) gene target primers. As a positive control was always included to preclude the possibility, and as this positive always amplified well, it was concluded that the negative results were an accurate reflection of *Bartonella* genome absence in the samples. Prevalence of this bacterial genus in the 171 Marion Island mice screened in this study was therefore 0 %.

Discussion

Molecular, sequence-based methods are useful techniques for the detection and identification of *Bartonella* species (Houpikian and Raoult 2001). *Bartonella* absence in Marion Island mice is unusual and may be attributed to (i) a founder effect, (ii) an inherently low level of infection in this species, or a combination of these two. Nucleotide sequencing of 91 individuals (Chapter 2) indicates that haplotype diversity is low for Marion Island *Mus*, indicating the likelihood of a single introduction and lending support to a founder effect. A prevalence estimate of approximately 50-60 % (Pretorius *et al.* 2004; Bastos 2007) has been recovered for rodent populations in South Africa. However, all studies that incorporated *Mus musculus* when screening for *Bartonella* recovered either no evidence of infection (De Sousa *et al* 2006; Ying *et al*. 2007; Chae *et al.* 2008, Morick *et al*. 2009), or a very low prevalence estimate (5.6 %) (Holmberg *et al*. 2003). The Holmberg *et al.* (2003) study isolated *B. grahamii* in one out of eighteen *Mus musculus* using partial *gltA* gene amplification and sequencing. It should however be stressed that all of the studies reporting a 0% prevalence had low sample sizes, ranging from 1-25 individuals. This study represents the largest, single species focussed study on *Bartonella* prevalence in *Mus musculus*.

Suggested further studies on *Bartonella* should focus on testing the susceptibility of the various *Mus musculus* subspecies to *Bartonella* infection to determine if the transmission rates among the *Mus musculus* subspecies differ. Mice have been experimentally infected with non-rodent *Bartonella* strains i.e. cat and human strains (Kabeya *et al.* 2003 and Infante *et al.* 2008, respectively). In the Kabeya *et al.* (2003) study, *B. henselae* was able to persist in the mice for up to 14 days. These findings show that mice could potentially transmit non-rodent *Bartonella* strains and might play a role in the maintenance of *Bartonella* in an environment where cats, mice and humans co-exist. However, in the Infante *et al.* (2008) study Balb/c mice were resistant to *Bartonella bacilliformis* regardless of the different routes and inoculation concentrations tested. These findings are not unusual as no reservoir other than humans has been found for *Bartonella bacilliformis* (Birtles *et al.* 1999). Given the negative status of the introduced

mice and the large number of mice screened from across the island, it is unlikely that *Bartonella* is harboured in this island population. However, the vectors of the infection, haematophagous ectoparasites should be evaluated.

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CHAPTER 6

General conclusion

Murid rodents are known to be reservoirs for many species of bacteria that cause diseases such as leptospirosis (*Leptospira interrogans*), endocarditis (*Bartonella elizabethae*), scarlet and rheumatic fever (*Streptococcus pyogenes*), and lyme disease (*Borellia burgdorferi*) just to name a few (Adler *et al*. 2002; Picard *et al*. 2004; Meerburg *et al*. 2009; Berglund *et al*. 2010). Despite this, studies evaluating the reservoir host potential of island-based *Mus musculus* are limited to a study on murine viruses on two Islands (Moro *et al.* 2003) and the assessment of three agents documented male bias, *viz.* encephalomyocarditis virus, *Leptospira* and *Streptococcus* on sub-Antarctic Marion Island (de Bruyn *et al.* 2008). The purpose of this study was to determine the genetic diversity of *Mus musculus* from Marion Island, as well as the prevalence of three bacterial disease agents *viz. Bartonella*, *Helicobacter* and *Streptococcus*. To this end, 171 mice collected in 2007 and in 2009 from seven geographical separate localities across Marion Island were assessed.

The first data chapter (Chapter 2) investigated the genetic diversity and origin of *Mus musculus* from Marion Island. To evaluate the genetic diversity of the mice, a sub population of 91 specimens representative of all sample localities were selected for characterisation. Amplification ~ 1.7 kbp fragment inclusive of cytochrome *b* (cyt *b*), tRNA-Thr, tRNA-Pro and the mitochondrial DNA control region was amplified and sequenced for 46 specimens. As no sequence variation was recovered for the cyt *b*, tRNA-Thr and tRNA-Pro regions, subsequent sequencing efforts were directed exclusively at the control region. The amplification of the control region generated a 539 bp product and resulted in the recovery of five Marion Island haplotypes. Four of the five haplotypes including the two previously published Marion haplotypes 1 and 2 (Van Vuuren and Chown 2007), were identified from the sixteen specimens sampled at Repettos (see fig. 4 in Chapter 2). Not only was haplotype 1 found at all the sampling

localities but it was also the most abundant haplotype occurring in $^{65}/_{91}$ mice sampled (see fig. 1 in General conclusion).

To determine the geographic origin of the mice, mtDNA sequences of seventeen *Mus musculus domesticus* sequences were downloaded from Genbank, as well as twenty five sequences from a sub-Antarctic study (see Hardouin *et al*. 2010) with two *Mus musculus musculus* sequences being used as an outgroup (see Table 1 in Chapter 2). The mtDNA sequences were selected to represent a geographic coverage of the global distribution of this *Mus musculus domesticus*. Subsequent phylogenetic analyses confirmed the finding of a preliminary study (Van Vuuren and Chown 2007) linking Marion Island mice to *Mus musculus domesticus* collected from Porto Santo, Madeira.

The bacterial prevalence of *Streptococcus* was the first of the three disease causing agents investigated (Chapter 3). The purpose of this chapter was to determine the potential disease transmission through possible urine contamination. In order to do this the kidneys were screened with *Lactobacillus, Streptococcus* and broad range bacterial universal 16S primers. As PCR uses preselected, species-specific DNA regions for the genotypic identification of bacteria (Alber *et al.* 2004), it is sensitive and reliable when using templates such as urine and renal tissue (Cameron *et al.* 2008).

A low *Streptococcus* prevalence of 7.4% was recovered, with $^{8}/_{9}$ samples grouping with 100% identity to a novel species first described in the de Bruyn *et al*. (2008) study. The remaining *Streptococcus* positive sample grouped with *Streptococcus parasanguinis* with 99% identity when the sequence was blasted against the Genbank database. The bacterial prevalence with respect to geographical locality can be seen in fig. 1. The higher prevalence in females (77.8%) *vs*. males (22.2%) found in this study contradicts the findings of previous studies (see Willoughby and Watson 1964; Medina *et al*. 2001), and may be attributable to a range of factors such as: increased male mortality due to *Streptococcus* infection; seasonal trapping biases; and increased female activity and exposure due to food provisioning for offspring. However, as the number of males caught in this study was found to be 74 whilst only 47 females were caught, it indicates that sexual bias in *Streptococcus* infection is more likely due to higher mortality rates for *Streptococcus*-infected males, resulting in fewer infected males being sampled.

As well as using molecular approach targeting the 16S rRNA gene, a culturing approach was undertaken to validate the potential novel species found. This was explored through four culturing attempts. Any bacterial growth recovered from the culture attempts were screened using a universal 16S primer set to identify the bacteria to genus level and where possible classify them to species level. *Staphylococcus* species were most commonly retrieved in culturing attempts, with only the non-zoonotic *Streptococcus salivarius* which is generally isolated from humans, being cultured successfully from the genus (see Appendix B in Chapter 3).

To continue with bacterial prevalence estimates, *Helicobacter* prevalence was investigated in chapter 4. As *Helicobacter* occur in the mucosa, gastro-intestinal tract (GIT) samples were taken, as well as liver samples as hepatic forms of *Helicobacter* are also known to occur (Fox *et al.* 1994; Patterson *et al.* 2000).

An overall *Helicobacter* infection rate of 12.4% was recovered with 86.7% of PCR-positive samples occurring in the GIT and 13.3% in the livers. A difference in geographical infection prevalence was seen with the highest prevalence occurring at the Cape Davis (37.5%), Repettos (18.8%) and Mixed Pickle (18.8%) sampling sites (see fig. 1). This was suggestive of geographical clustering to the northern part of the island which indicates a possible host population infection rate difference in Marion Island mice, with respect to this bacterial genus. The majority of *Helicobacter* species were found in males (66.7%) with only five females (33.3%) being infected, which may be an artefact of sampling bias as more males were sampled than females.

In this study two species were recovered that correspond to previously described species *viz. H. hepaticus* and *H. typhlonius*; however, the remaining *Helicobacter* positive samples appear to represent novel species. As *H. hepaticus* colonises the lower bowel of mice, it can be shed in the faeces promoting horizontal transmission through the faecal-oral route (Whary and Fox 2006), potentially posing a zoonotic threat to other mammals on Marion Island.

The last data chapter (Chapter 5) investigated the presence of *Bartonella* in Marion Island mice. The 0% infection prevalence obtained in this study, contrasts markedly with the generally high levels of prevalence reported for other invasive rodent species (Ellis *et al.* 1999). However, all studies on *Mus musculus* recovered either no

evidence of infection (Morick *et al*. 2009), or a very low prevalence estimate (Holmberg *et al*. 2003).

Overall, this study represents the first bacterial prevalence investigation in *Mus* from Marion Island that utilises a large sample size and geographically separate sample localities. The presence of several potentially zoonotic bacterial genera supports future research in this field, with the aim to detect and prevent large mammal die-offs as experienced in 2007 (de Bruyn *et al*. 2008). Future studies should focus on a wider variety of potential zoonotic bacteria as well as the role of ectoparasites on mice in Marion Island.

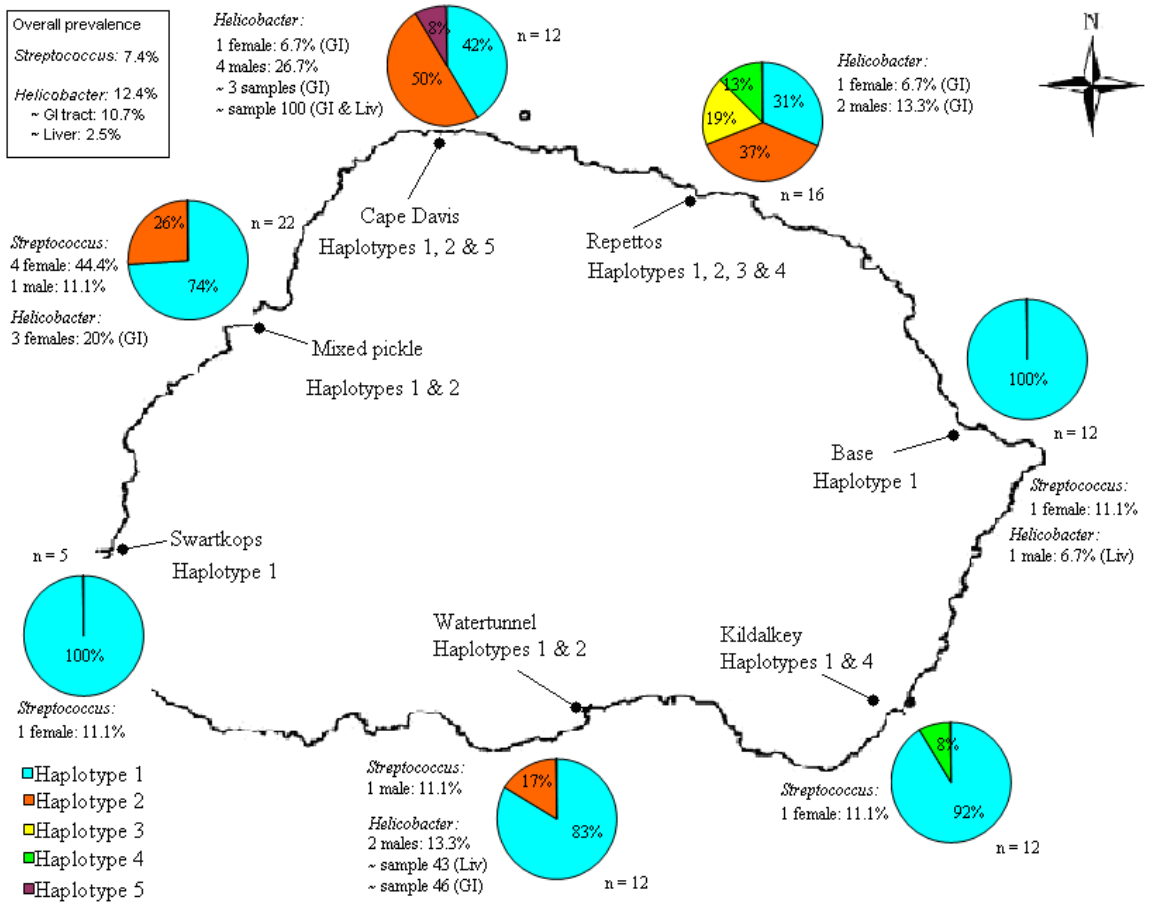

Fig. 1 Map summarising haplotype diversity and *Streptococcus* and *Helicobacter* prevalence and diversity with respect to *Mus musculus* sampling locality on Marion Island.

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