Molecular detection of bovine pathogens and microbiota harboured by *Stomoxys calcitrans* occurring in South African feedlots

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RESEARCH OUTPUTS

CONFERENCE PAPER

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ABSTRACT

Stomoxys calcitrans are biting flies commonly known as stable flies. They belong to the family Muscidae which is composed of 18 described species under the genus *Stomoxys* with a cosmopolitan distribution. *Stomoxys* spp. are of economic importance worldwide due to their ability to mechanically transmit various pathogens including bacteria, viruses, and protozoa. Vector borne lumpy skin disease virus, rickettsiosis, anaplasmosis and ehrlichiosis are major diseases that threaten and affect livestock production in tropical and sub-tropical regions of Africa. Some bacteria are essential for the larval survival and development for stable flies, but little is known about the innate microbial communities of stable flies. The aim of this study was to characterize stable flies inhabiting selected feedlots in South Africa and detect disease-causing pathogens they are harbouring using PCR. Furthermore, this study utilized Illumina MiSeq next generation sequencing of 16S amplicons to characterize stable fly microbiome.

A total of 10195 stable flies were collected from three feedlots, with 9993 from Van der Leeuw Boerdery in Potchefstroom, North West province, 175 from GysbertHoek in Sasolburg, Free State province, and 27 from Doornbult in Limpopo province. Morphological identification of stable flies was further supported by amplification of *CO1* and *16S rRNA* genes whereby their sequences matched with respective stable fly genes on NCBI database. Furthermore, phylogenetic analysis of *CO1* gene also showed that *Stomoxys calcitrans* characterized in this study clustered with other *Stomoxys* spp. from around the world.

PCR detected *Anaplasma marginale* infections from *S. calcitrans* with infection rates of 10% and 16% in flies from Free State and North West respectively, whilst none of the flies from Limpopo were positive for the presence of *A. marginale*. This study is the first to report on the detection of *A. marginale* infections in stable flies by PCR in South Africa. The current study has detected 27% LSDV by PCR from *S. calcitrans* collected from North West alone**.** None of *Rickettsia* and *Ehrlichia* spp. were detected from all the sampled provinces.

This study also attempted to determine the best washing method for the removal of microbes from the fly's surface by washing in 70 % ethanol, 10% bleach and 10% tween 20. In the current study, 70% EtOH was one of the less effective disinfecting methods

tested, while 10% bleach and 10% tween20 solutions appeared to be the most effective methods of sterilizing the external surface of stable fly without interfering with the analysis of the mitochondrial DNA of fly internal contents.

Analysis of gut microbes from 50 South African *Stomoxys* flies produced a total of 462 operational taxonomic units (OTUs). The most abundant genera at Van der Leeuw Boerdery, Potchefstroom was *Sphingomonas* at 12.1%, followed by *Wolbachia* at 11.7%. At GysbertHoek, Sasolburg, the most abundant genera were *Sphingomonas* at 13.4 %, followed by *Agrobacterium* at 3.2%. Bacterial genera of medical, veterinary and ecological importance detected in the current study include *Clostridium, Bacillus, Anaplasma, Rickettsia, Wolbachia, and Rhizobium*.

Keywords: Stable fly, *Stomoxys calcitrans*, *Ehrlichia* spp, Lumpy skin disease virus, *Anaplasma marginale*, *Rickettsia* spp, metagenomics, Feedlot.

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING	ABBREVIATION	MEANING
CO1	Cytochrome Oxidase 1	SFG	Spotted Fever Group
16S rRNA	Ribosomal Ribonucleic Acid	DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction	SEM	Scanning Electron Microscope
NCBI	National Centre for Biotechnology Information	propan-2-ol	Isopropyl alcohol
OTU	Operational Taxonomic Unit	CO_2	Carbon Dioxide
LSD	Lumpy Skin Disease	*gltA*	Citrate Synthase encoding gene
RMSF	Rocky Mountain Spotted Fever	Tris-HCl	Trisaminomethane hydrochloric Acid
ATBF	African Tick Bite Fever	EDTA	Ethylenediamine Tetraacetic Acid
NGS	Next Generation Sequencing	SDS	Sodium Dodecyl Sulphate
MEGA7	Molecular Evolutionary Genetics Analysis version 7	Pro-K	Proteinase K
PBS	Phosphate-Buffered Saline	NaCl	Sodium Chloride
LSDV	Lumpy Skin Disease Virus	dNTP	Deoxyribonucleotide Triphosphate
OIE	World Organization for Animal Health	KCl	Potassium Chloride
CME	Canine Monocytic Ehrlichiosis	$MgCl_2$	Magnesium Chloride
HME	Human Monocytic Ehrlichiosis	QG buffer	Solubilization buffer
HEE	Human Ewingii Ehrlichiosis	QC buffer	Wash buffer
EB Buffer	Elution Buffer	mtDNA	Mitochondrial Deoxyribose Nucleic Acid
ml	Milliliter	nM	Nanomolar
µl	Microliter	CSV	Comma Separated Values

PANDAseq	Paired-end Assembler for Illumina Sequences	pH	Potential of Hydrogen
RPM	Revolution Per Minute	DDH_2O	Double Distilled Water
FASTA	Fast Alignment Search Tool	QIIME	Quantitative Insights into Microbial Ecology
BLASTn	Basic Local Alignment Search Tool	EtOH	ethanol
MAFFT	Multiple Alignment Fast Fourier Transform	MRS	MiSeq Reporter Software
ML	Maximum Likelihood	OBP	Onderstepoort Biological Products

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CHAPTER 1

INTRODUCTION

Stomoxys calcitrans (Diptera: Muscidae) commonly referred to as stable fly is an obligate blood sucking insect (Yeruham *et al*., 1995; Muenworn *et al*., 2010; Scully *et al*., 2017), which is considered as economically significant pest of livestock worldwide (Muenworn *et al*., 2010; Baldacchino *et al*., 2013). It is the primary livestock pest in the United States with estimated economic losses of up to US$2 billion annually (Taylor *et al*., 2012; Kneeland et al., 2013). Both female and male stable flies are blood feeders (Foil & Hogsette, 1994; Muenworn *et al*., 2010; Tangtrakulwanich, 2012), and are biological vectors of nematodes (Holdsworth *et al*., 2006), and mechanical vectors of blood infecting parasites such as *Besnoitia* spp., *Anaplasma* spp., and Lumpy Skin Disease virus which are important pathogens in South African feedlots (Tuppurainen *et al*., 2013a). The flies feed on a wide range of animals, especially livestock and wild animals, and sometimes-even humans when their preferred host is unavailable. Preferred biting sites are the lower parts of their host, legs of horses and cattle (Yeruham *et al*., 1995). The direct effect of their bite is development of necrotic dermatitis on their host (Salem *et al*., 2012).

Blood feeding on hosts is very important for these flies as the blood provides all the nutrients that they need for development and reproduction (Schofield & Torr, 2002). Mechanical transmission is of high importance as it is the most threatening “indirect effect” of the blood-feedinng insects which occurs through either contamination of mouthparts or regurgitation of digestive tract contents (Butler *et al*., 1977; Doyle *et al*., 2011). The *Stomoxys* spp. are considered as important mechanical vectors of various microorganisms because of their feeding habits (Baldacchino *et al.,* 2013). When feeding, *S. calcitrans* administers a painful bite to their host, in trying to avoid the bite the cattle will stomp their feet, throw their heads, twitch their skin, swish the tail and bunch together (Dougherty *et al*., 1993; Mullens *et al*., 2006). The time and energy that the cattle spend on these avoidance strategies, is the time lost on their feeding which impacts on their weight gain. The feeding deficiency of cattle can increase due to high stable fly infestation; however, cattle become less sensitive and more adaptive to the bite when the stable flies feed in high numbers after which the weight loss is less

affected (Catangui *et al*, 1997; Campbell *et al*., 2001). According to Erasmus (2015) the cattle also compensate for the feeding interference during the day by feeding in the late hours of the night and early hours of the day of the morning when flies are not active (Catangui *et al*., 1993).

1.2 Statement of the problem

Stable flies (*S. calcitrans*) have major economic and health effects on both livestock and human beings**.** They attack agricultural animals and sometimes even human beings throughout the world to feed on their blood (Salem *et al*., 2012; Muller *et al*., 2012). Stable flies give a painful and irritating bite to the host and this blood feeding behaviour results in the loss of blood, weight, and milk production of the cattle. The painful bite from stable flies also cause annoyance and skin irritation to the host (Baldacchino *et al*., 2013), and sometimes causing death.

Stable flies have other indirect impacts on affected countries by transmitting pathogens to livestock, which affects their health and ultimately results in decreased production (Mihok *et al*., 1996; Scoles *et al*., 2005; Baldacchino *et al.,* 2013). The infestation of *S. calcitrans* on livestock costs millions of dollars per year of losses in the cattle industry to the United Stated and other affected countries (Salem *et al*., 2012). Stable flies also carry valuable microbes that enhance their general physiological capacities, and a good number of these insects also convey and transmit microbes that are pathogenic to their host (Perilla-Henao & Casteel, 2016). The vast majority of stable flies use blood as a sustenance source and typically acquire pathogens while feeding on infected host and pass on the disease-causing agents to other hosts during the course of subsequent meals (Weiss & Aksoy, 2011). Stable flies also affect the cattle industry by damaging the hides of cattle due to the holes created by the piercing of the skin during feeding (Bishop, 1913; Catangui *et al*., 1993; Cook *et al*., 1999).

Lumpy skin disease (LSD) is known to be endemic in the African continent (Babiuk *et al*., 2008; Abutarbush *et al*., 2015; Tuppurainen *et al*., 2013b; Al-Salihi, 2014). The first reported cases of LSD in South Africa were in the North West Province in 1944 (Hunter & Wallace, 2001). The disease occurred as a panzootic infecting 8 million cattle and was recorded continuously until 1949, where it resulted in great economic loses (Hunter & Wallace, 2001; Al-Salihi, 2014). The major spread of LSD is due to the transportation

of cattle between farms, drinking contaminated water from common water routes (Tuppurainen *et al*., 2013a; Al-Salihi, 2014), and in all likelihood by means of insect vectors (Tulman *et al*., 2001; Chihota *et al*., 2003; Babiuk *et al*., 2008; Abutarbush *et al*., 2013; Tuppurainen *et al*., 2013a). The common stable fly, the *Aedes aegypti* mosquito and some African tick species of genus *Rhiphecephalus* and *Amblyomma* have been associated with the transmission of the virus (Tuppurainen *et al*., 2013a; 2013b; Lubinga *et al*., 2014a). The mechanical spread of the LSD infection is primarily associated with biting insects, and all field observations by Al-Sahili (2014), confirmed that the epidemics of LSD occur at periods of high biting insect activity. Research on vectors and epidemiology of LSD in South Africa is essential for better disease control in the country.

Bovine anaplasmosis caused by *Anaplasma marginale* is widely distributed around the world, and is endemic in South Africa (Mutshembele *et al*., 2014). As estimated by de Waal (2000), 99% of the total cattle population is at risk of acquiring *A. marginale* infection. Studies conducted by Mtshali *et al*., (2007) recorded a 60% prevalence of *A. marginale* from cattle in the Free State Province alone. Five tick species, namely *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi*, *R. simus* and *Hyalomma marginatum rufipes,* have been implicated in the transmission of *A. marginale* in South Africa (Hove *et al*., 2018). This pathogen can also be transmitted mechanically by biting insects such as stable flies and blood contaminated fomites (Mutshembele *et al*., 2014). Most studies have focused on *Anaplasma* – tick relationship in South Africa (Mutshembele *et al*., 2014; Mtshali *et al.,* 2015; Chaisi *et al*., 2017; Mtshali *et al*.,2017; Hove *et al*., 2018), whilst there is little to none which have comprehensively examined the *Anaplasma* - stable fly relationship.

Rickettsiosis caused by *Rickettsia conorri* (Mediterranean spotted fever [MSF]) and *Rickettsia africae* (African tick bite fever [ATBF]) are the most common forms in the sub-Saharan Africa. Although not commonly reported among indigenous people because they do not display clinical signs of the disease (Kelly, 2006; Rutherford *et al*., 2004), *Rickettsia* have however proven to be problematic to South Africa's tourism industry, where numerous reports of illness and infection have been reported by the tourists returning to their home countries after visiting nature reserves in South Africa (Portillo *et al*., 2007; Raoult *et al*., 2001; Roch *et al*., 2008).

Erhlichiosis is a much-diversified disease, but organisms causing infection in South Africa are yet to be isolated and characterized. Serological studies have shown that up to 75% of dogs have significant antibody titres against *Erhlichia canis and E. chaffensis* in Bloemfontein, South Africa (Pretorious & Kelly, 1998). DNA of *E. canis* and that of a novel *Erhlichia* species closely related to *Erhlichia ruminantium* have been found in the blood of dogs in South Africa. These animals showed clinical signs suggestive of erhlichiosis, but it could not be confirmed whether the *E. ruminantium*-like organism was the cause of the illness (Allsop & Allsop, 2001; Inokuma *et al*., 2005; McBride *et al*., 1996). Recently, Mtshali *et al*., (2017) and Ringo *et al*., (2018) have confirmed the occurrence of *E. ruminantium* in tick and livestock in South Africa. However, there is a lack of studies to determine the *Erhlichia*-stable fly relationship in South Africa.

According to Azambuja *et al*., (2005), numerous insects contain vast groups of diverse microorganisms that most likely surpass the quantity of cells in the bug itself. Little is known about the relative niches occupied by the parasites and the microbiota in various compartments of the vector's digestive tract, so there is a need to consider the colonization of the gut following co-infection of insect vector (Azambuja *et al*., 2005). All insects' studies to date indicate resident microorganisms and, although some insect taxa are not obligatory subject to their microbiota, there is expanding proof that these micro-organisms impact numerous insect trait (Douglas, 2014). Metagenomics is the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and laboratory cultivation of individual species (Kim *et al*., 2013; Finney *et al*., 2015). Metagenomics gives access to practical gene framework of microbial communities. The Next Generation Sequencing (NGS) platforms such as Illumina MiSeq sequencing provides exciting new opportunities in biomedicine, offering novel and rapid methods for whole genome characterization and profiling (Duan & Cheng, 2017). These high-throughput sequencing techniques overcome the defects of traditional sequencing methods, such as read frames and larger error, and can elucidate on microbial community information (Frey *et al*., 2014)

There is a great number of studies and available information on ticks and tick-borne diseases in South (Mtshali *et al*., 2004).However, there is lack of detailed studies on the

role played by stable flies in transmission of pathogens to livestock, which has resulted in little or no information on the relationship of stable flies (vector) and microbial pathogens. As a result, the current study was aimed at filling in this information gap and determining whether there is a relationship between LSD virus, *Anaplasma* spp., *Rickettsia* spp., and other bacterial pathogens with *S. calcitrans* at feedlots in the Free State, North West and Limpopo Provinces. The study further seeks to determine genetic diversity of the South African *S. calcitrans* in comparison to other related species around Africa and the world.

1.3 Research hypothesis

Stable flies harbour a variety of bacterial pathogens of veterinary and economic importance as well as the LSD virus.

1.4 Aims and objectives

1.4.1 Aim of the study

The aim of this study is to conduct morphological and genetic characterization of stable flies (*S. calcitrans)* found in feedlots and to detect microbial pathogens of cattle that they are possibly harbouring.

1.4.2 Objectives of the study

- To identify stable flies using morphological and genetical analysis.
- To conduct phylogenetic analysis of stable fly's *CO1* and *16S rRNA* genes using MEGA7 software.
- To detect LSD virus, *Anaplasma* spp., *Rickettsia* spp. and *Ehrlichia* spp. harboured by stable flies using PCR.
- To determine an effective method for washing stable flies to remove externally attached microbes.
- To detect bacterial communities associated with stable flies by 16S metabarcoding using next generation sequencing.

1.5 Outline of dissertation

Chapter 1 - Introduction:

Provides a background on stable flies, statement of the problem, aim, objectives and hypothesis.

Chapter 2 – Literature review:

Reviews the classification of stable flies, their life cycle and role in transmission of pathogens. Furthermore, the chapter introduces lumpy skin disease, anaplasmosis, erhlichiosis and ricketssiosis as diseases of economic importance in cattle. The use of metagenomics as culture independent tool for detection of bacterial communities in arthropods is further highlighted in this chapter.

Chapter 3 - Materials and methods:

Gives a detailed description of the study approach including, description of the study areas, materials used and methods followed, as well as how data was analyzed.

Chapter 4 – Results:

A representation of the data obtained in this study.

Chapter 5 – Discussion, conclusion and recommendations:

The interpretation of data with conclusions showing whether the aims and objectives of the study have been achieved as well as recommendations for further action and studies that needs to be undertaken with references to data obtained from this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology of *Stomoxys calcitrans*

2.1.1 Taxonomy

Muscidae is a large dipteran family that contains some 450 described species from 180 genera (De Carvalho *et al*., 2005). Family is divided into seven subfamilies following the classification proposed by De Carvalho *et al*., (1989). The subfamily Muscinae, which is considered to be among the most basal subfamilies comprises the Muscini and Stomoxyini tribes (De Carvalho *et al*.,1989; De Carvalho,2002). The tribe Muscini has a worldwide distribution and exhibits a wide diversity in both morphology and ecology including their reproductive strategies and the feeding habits of the larvae and adults. Based on its feeding habits (blood feeding), the tribe Stomoxyini consists of the genus *Stomoxys* which encompasses 18 described species. The classification of *Stomoxys* is as follows (Zumpt, 1973):

Kingdom: Animalia Linnaeus, 1758

Phylum: Arthropoda von Siebold, 1848

Subphylum: Hexapoda Latreille, 1825

Class: Insecta Linnaeus, 1758

Subclass: Pterygota Lang, 1888

Order: Diptera Linnaeus, 1758

Suborder: Brachycera Linnaeus, 1758

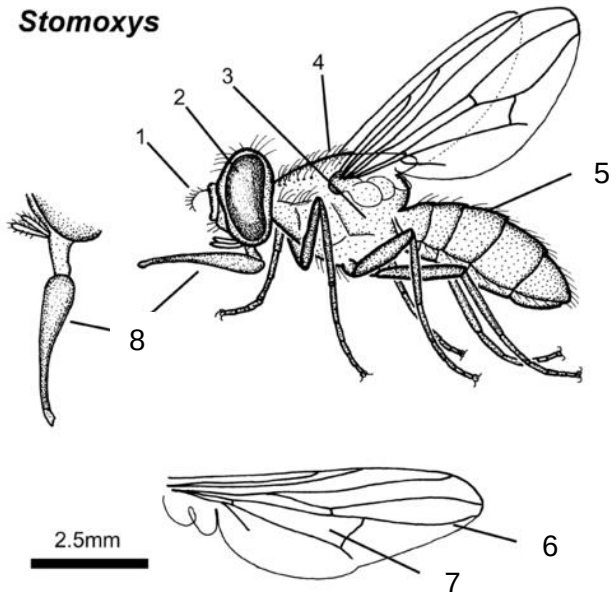
Family: Muscidae Latrielle, 1808

Tribe: Stomoxyini Meigen, 1824

Genus: *Stomoxys* Geoffroy, 1762

2.1.2 Morphology

Stomoxys flies (Diptera: Muscidae) are in same family as house flies and can be distinguished from other muscoid flies by piercing and sucking proboscis that projects from the front of the head (Figure 2.1) (Masmeatathip *et al*., 2006). General characters of *Stomoxys* genera include grey thorax with four longitudinal dark stripes and a broad abdomen with dark spots on the second and third segments of the abdomen (Howel *et al*., 1978; Foil & Hogsette 1994; Masmeatathip *et al*., 2006; Tangtrakulwanich 2012). The flies are often found breeding in outdoor silage and animal manure (Foil & Hogsette 1994)



1. antennae
2. compound eye
3. thorax
4. setae
5. abdomen
6. wing vein 4 (curved end)
7. wing cell (symmetrical trapezoid discal)

Figure 2.1: Adult *Stomoxys* morphological illustration indicating mouthparts and wings (en.wikibooks.org)

There are 18 species of *Stomoxys* described worldwide (Foil & Hogsette 1994; Keawrayup *et al*., 2012), which include *S. calcitrans, S. indicus, S. sitiens, and S. bengalensis* whom their common characters are described as follows: Adult body length ranges between 5 - 7 mm long (Masmeatathip *et al*., 2006). Antenna has arista with short setae, piercing and sucking proboscis with short maxillary palps, that protrudes from the front of the head (Foil & Hogsette, 1994). Eyes are large and brownish in color. Thorax has a hypopleuron without a row of stout setae. Thorax is colored black with prominent dorsal-longitudinal grey stripes. Abdomen has a mottled pattern of grey and

black color dorsally, but a pale or yellow color ventrally. The wing has vein 4 that curves evenly toward the lower outermost edge (Zumpt, 1973) and a discal cell that is symmetrical, trapezoid in shape. Body length ranges from 4 - 7 mm, easily recognized by one marked median spot and two lateral dark round spots presented on the second and third abdominal segments. Width of frons at its narrowest point measuring 1/3 or more of eye length. Thorax and abdomen are dark grey and olive-brown, with distinct pattern. Wing hyaline, terminal part of r1 not setulose. Legs are dark with only bases of tibiae being more or less extensively pale (Zumpt, 1973)

2.1.3 Reproduction

The stable fly breeds in a number of different habitats commonly found in agricultural areas such as, silage, livestock manure, and rubbish dumps. (Bishop, 1913; Hunter & Curry, 2001). Figure 2.2 shows the basic life cycle of stable flies whereby eggs take up to four days to develop based on temperature and humidity. Extreme temperature appears to reduce the duration of the egg production. It also depends on the length of days that the egg was kept inside the female. The larval developmental stage lasts up to 30 days, based on how befitting the habitat will be and on the availability of food. The third instar will then pupate for 6 to 20 days. The length and development of the pupa is based upon food availability during larval growth (Bishop, 1913).

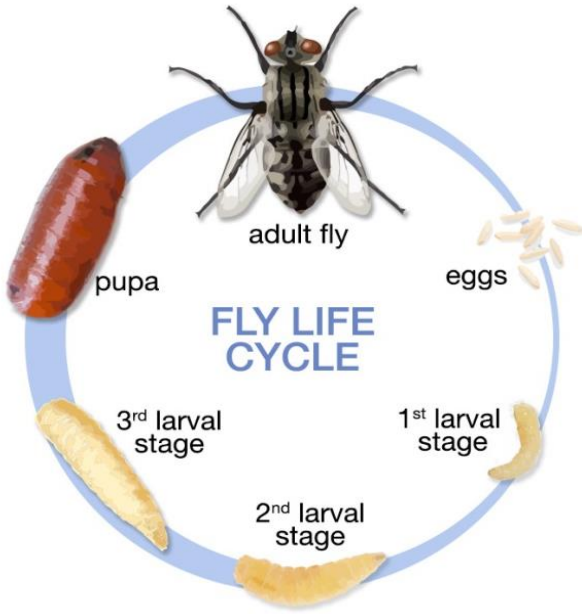


Figure 2.2: Life cycle of *Stomoxys calcitrans* (Linneaus, 1758). Image adopted from Patra *et al*., (2018).

The female must be fully fed with blood for development and reproduction of eggs. The females keep their eggs retained up until their third feed, the eggs will only be laid if the female could be fully fed at least four times (Bishop, 1913). The female deposits the eggs on livestock moist manure and can lay between 60 to 130 eggs during one cycle (Foil & Hogsette, 1994). The males die soon after mating and the females die after oviposition.

2.2 Distribution of *Stomoxys calcitrans*

Among the *Stomoxys* spp.*, S. calcitrans* is the only cosmopolitan spp. (Muenworn *et al*., 2010; Baldacchino *et al*., 2013). All the other species are exclusively tropical. According to Masmeatathip *et al*., (2006), a total of 12 of the 18 species are distributed on the African continent, four on the Asian continent, and one has been reported on both the African and the Asian continents. Marquez *et al*., (2007) conducted a stable fly phylogeny with the use of mitochondrial DNA and *16S rDNA* which explained the geographic origin of the new world *S. calcitrans* population which led to the conclusion that the most parsimonious scenario is that the new world *Stomoxys* flies had a Palearctic origin within the past 500 years. Ecological evidence suggests that stable flies are strongly vagile and can disperse far and wide (Eddy *et al*., 1962). The stable fly generation is principally dependent on temperature, favourable hot and humid climate that makes them successful in their distribution (Dsouli-Aymes *et al*., 2011). In their normal habitat, in livestock facilities, the stable flies are not usually problematic to human beings. However, certain regions like the U.S. have a condition that results in stable flies attacking human beings (Muenworn *et al*., 2010). Stable flies are found in rural areas near stables, slaughterhouses, cattle markets, rubbish dumps, and locations related mainly to the presence of fermenting organic material (Muenworn *et al*., 2010).

2.3 Economic, medical and veterinary importance of *Stomoxys calcitrans*

2.3.1 Economic importance

Because of their wide distribution, and their annoyance to both man and livestock, their effects as pest on the cattle industry is of primary importance. Stable flies are a standout amongst the most serious insect pests of feedlots and dairy cattle in summer months in the United States (Kneeland *et al*., 2013). The most evident impact of flies is

the change in behaviour of cattle when under attack. When stable flies occur in large numbers on cattle, every animal attempt to protect their front legs (the preferred feeding site of the flies) by stamping, tail swiping, skin twitching, and head throwing (Mullens *et al*., 2006). It is estimated that the fly ration per front leg to the rest of the animal is 2.8:1 (Berry *et al*., 1983). Furthermore, the economic injury level for feeder cattle is when the stable fly population reaches an average of five flies per front leg (Mcneal & Campbell, 1981). Due to the increase in the use of large round hay bales to feed animals, where a significant amount of wasted hay is mixed with manure and creates an excellent habitat for larval development, cattle become more exposed to stable flies. Severe biting activity can result in reduced weight gain and milk production (Baldacchino *et al*., 2013), and may also damage the hides of cattle due to the holes created by the piercing of the skin during feeding (Bishop, 1913; Catangui *et al*., 1993; Cook *et al*., 1999).

Locations related mainly to the presence of fermenting organic material and manure around bales are a primary source of stable flies in late spring (Taylor & Berkebile, 2008). Stable flies also cause important economic loss when they aggregate in large number in tourist areas such as in the Great Lakes area (Newson, 1977), Florida coast (King & Lernnet, 1936) and New Jersey seaboard (Hansens, 1951) where reports of evacuation had taken place due to stable fly attacks (Albuquerque, 2014).

2.3.2 *Stomoxys calcitrans* as vector of pathogens

Stable flies have been implicated as mechanical and biological vectors of disease-causing pathogens such as bacteria, protozoa, helminths and viruses.

2.3.2.1 Mechanical transmission of pathogens by *Stomoxys calcitrans*

Mechanical transmission is one of the indirect effects that arises from hematophagous insects feeding mode. It occurs through contamination of mouthparts during feeding on an infected host or regurgitation of digestive tract substance that contain infective material (Baldacchino *et al*., 2013). The painful bite associated with the flies leads to the defensive movements by the host animal. The fly may then take off and land on another animal and exchange pathogens remaining in its mouth parts from its previous meal to a susceptible animal (Chihota *et al*., 2003). The transmission of pathogens occurs through the saliva that is injected by hematophagous flies prior to blood sucking.

Furthermore, it has been demonstrated that stable flies can regurgitate some part of a previous blood meal before taking up another and this could be an important way of transmitting high doses of disease agents (Straif *et al*., 1990). However, this phenomenon is limited by the short survival of pathogens which may be inhibited by digestive secretion. Stable flies are frequent feeders with variable intervals between blood meals ranging from 4 to 72 h. Thus, the crop regurgitation of infectious blood could easily establish in these flies. Possible pathogens transmitted by tabanids have been reviewed by several authors and proved to be as varied as bacteria, viruses, and parasites (Foil, 1989; Krinsky, 1976).

2.3.2.2 Biological transmission of pathogens by *Stomoxys calcitrans*

The *S. calcitrans* is an intermediate host of the nematode *Habronema microstoma*, (family Spirudidae). The adults of *H. microstoma* occur in the stomach of horses, under a layer of mucus and can cause inflammation of mucosa, digestive disturbances, and even chronic gastritis and ulceration (Zumpt, 1973*;* Baldacchino *et al*., 2013). The embryonated eggs that hatch in the fecal mass are ingested by maggots (*Musca* or *Stomoxys* larvae), within which they develop to the infective L3 stage. The infective stage is achieved when the stable fly imago emerges after pupation. The infective larvae are kept on the nostrils, lips, eyes, or injuries of the host when the stable flies take their blood meal. Larvae around nostrils and lips are swallowed and develop in the stomach of host (Traversa *et al*., 2008). Larvae deposited on mucous membranes (vulvae, prepuce, eye) or on injured tissues cannot complete their life cycle; however, they induce a local inflammatory reaction with strong eosinophilia causing cutaneous ‘‘summer sores’’ and/or ophthalmic habronemiasis (Anderson, 2000).

2.4 Control of stable flies

The control of *S. calcitrans* in stables as well as in the field has become of utmost importance over the past years because of their effect on the profit margin, their ability to transmit pathogens and their nuisance to human beings in houses and outdoor camps (Baldacchino *et al*., 2013;Kneeland *et al*., 2013). Various control measures such as chemical compounds, traps, ecological modification of the environmental conditions,

utilization of parasites and predators, and feedlot management strategies are possible control options. Major effective control measures of stable flies include preventing breeding by removing rotting straw and manure, kill larvae before they become adults and start producing eggs, and the use of pesticides and repellents (Foil & Hogsette, 1994). Beneficial organisms such as predators, parasites and natural competitors occur naturally in similar breeding locations of the stable fly larvae (Zumpt, 1973). They play an important role in regulating the densities of stable fly populations. These organisms attack the eggs, larvae, and pupae of the stable flies providing natural control (Foil & Hogsette, 1994). The possibility of biological control by mass rearing of parasitoids could also be an option. The use of traps such H-traps and/or Vavoua traps, are also effective in collecting flies.

2.5 Diseases of interest in the current study

2.5.1 Anaplasmosis

The genus *Anaplasma* (Rickettsiales: Anaplasmaceae) incorporates three species that infects ruminants; *Anaplasma marginale, A. centrale, and A. ovis* (De La Fuente *et al*., 2005). Bovine anaplasmosis is an arthropod-borne disease caused by *A. marginale*, while *A. ovis* is a pathogen of sheep and is not infectious to cattle. The *A. centrale* is a less pathogenic life form which is utilized as live vaccine for livestock in Israel, South Africa, South America and Australia (Kocan *et al*., 2003, De La Fuente *et al*., 2005). The *A. marginale* infection was first described by Sir Arnold Theiler in the erythrocytes of South African cattle as "marginal points" and was later corroborated by Salmon & Smith in 1896, who described the presence of a point-like pathogen in blood smears of cattle as "very minute roundish body which is stained blue to bring it into view". It is grouped within the order Rickettsiales, which was as recently renamed into two families, Anaplasmataceae and Ricketsiaceae based on genetic analysis of *16S rRNA*, *groEL* and surface protein genes (Dumler *et al*., 2001). This bacterium is a gram-negative rickettsia (Bastos *et al*., 2015) that infects a wide range of animals, including humans (Rar & Golovljova, 2011). Members of the family Anaplasmataceae are obligate intracellular living organisms discovered solely inside membrane bound vacuoles in the host cell cytoplasm (Rodríguez *et al*., 2009, Rar & Golovljova, 2011). With a specific end goal to persist in nature, *A. marginale* infects the mammalian host which normally

remains infected serving as reservoir host of infection through ticks (Rodríguez *et al*., 2009). Tick-borne diseases such as anaplasmosis constitute a constraint for livestock production and are the cause of major health and management problems and economic impact (Rodríguez *et al*., 2009). Biological transmission of *A. marginale* by its tick vectors is not dependent on the level of rickettsemia infection in the host and is considered to be necessary for transmission from persistently infected carriers. Within the tick, *A. marginale* replicates both within the gut epithelium and in the salivary gland acini, culminating in levels of 10^4 – 10^5 organisms per salivary gland during subsequent transmission (Lohr *et al*., 2002; Futse *et al*. 2003). Replication within the tick results in similarly high levels of *A. marginale* in the salivary gland regardless of the rickettsemia level in the blood during acquisition feeding (Eriks *et al*., 1993). In contrast, transmission by biting flies is purely mechanical and thus directly dependent on the level of rickettsemia during feeding. Because of this, fly-borne mechanical transmission is thought to be possible only during the acute phase of infection. Although mechanical transmission of *A. marginale* by biting flies is commonly assumed to be a component of the epidemiology of anaplasmosis in some areas of the United States, neither the quantitative parameters of fly-borne transmission nor its efficiency relative to tick-borne transmission have been reported.

2.5.1.1 Economic impact

Bovine anaplasmosis leads to high morbidity and mortality rates in susceptible cattle, which causes great economic losses in affected countries (Rymaszewska & Grenda, 2008). Several parameters are used to measure the losses that are due to anaplasmosis and they include: decreased weight gain, low milk production, abortions, mortality, and the treatment costs of anaplasmosis (Eriks *et al*., 1989;Radwan *et al*., 2013). In trying to determine the exact annual losses caused, control studies have been carried out in countries most affected by anaplasmosis. The current loss in livestock production as a result of anaplasmosis morbidity and mortality in the United States are estimated to be over $300 million per year, whereas in Latin America those losses were calculated to be approximately $800 million (Rymaszewska & Grenda, 2008).

2.5.1.2 Geographic distribution of anaplasmosis

Anaplasmosis occurs in tropical and sub-tropical areas throughout the world (Rodriguez *et al*., 2009). In the United States, anaplasmosis is enzootic throughout the southern Atlantic states, Gulf Coast states, and several of the Midwestern and Western states. However, anaplasmosis has been reported in almost every state in the United States, and this widening distribution may be due to increased transportation of cattle and hence the opportunity for mechanical transmission from asymptomatic persistently infected cattle. Anaplasmosis is endemic throughout most of South Africa (Mutshembele *et al*.,2014) and Namibia, except in the low-rainfall areas where tick populations are minimal. As estimated by De Waal (2000), 99% of the total cattle population are at risk of acquiring *A. marginale.* Tick vectors such as *Rhipicephalus decloratus*, *R. microplus*, *Hyolomma marginatum rufipes*, and *R. simus* have been implicated in the transmission of *A. marginale* in South Africa (Mtshali *et al*., 2007), however various hematophagous flies such as *S. calcitrans* and blood contaminated fomites can mechanically transmit the pathogen (Aubry & Geale, 2011). The current study was formulated to demonstrate whether there is a relationship between feedlot stable flies and *A. marginale* in South Africa.

2.5.2 Lumpy skin disease virus

Lumpy skin disease virus (LSDV) is the causative agent of Lumpy skin disease (LSD), a pox viral disease of cattle which belongs to the genus Capripoxvirus, subfamily chordopoxvirinae and the family Poxviridae. (Babiuk *et al*., 2008). The most common mode of transmission of LSDV is through mechanical transmission by hematophagous vectors (Lubinga *et al*., 2013) such as mosquitos *Aedes aegypti* (Chihota *et al*., 2001), and biting flies *S. calcitrans*, (Chihota *et al*., 2001). Transmission can also occur through direct contact of drinking troughs or food that is contaminated with infected saliva or respiratory secretions (Lubinga *et al*., 2013), and contaminated needles (Davies, 1991). Lumpy skin disease virus can also be isolated from bovine milk and semen (Irons *et al*., 2005). Although LSDV is not associated with high mortality rates, it has major socio-economic impacts in endemic countries (Coetzer, 2004). It is endemic in African continent and the Middle East and poses a great threat in spreading to Asia and Europe (Tuppurainen & Oura, 2012). There are serious economic constraints of

livestock production in countries that are affected, causing a decrease in milk production and weight gain (Weiss, 1968), infertility on both male and female livestock, and abortions. LSD outbreaks have significant indirect economic impacts by restricting the international trade of live animals and animal products from endemic countries (Babiuk *et al*., 2008).

2.5.2.1 Pathogenesis

Subcutaneous or intradermal inoculation of cattle with LSDV results in the development of a localized swelling at the site of inoculation after four to seven days and enlargement of the regional lymph nodes while generalized eruption of skin nodules (Figure 2.3) usually occurs seven to 19 days after inoculation (El-Bagoury, 2012). In experimentally infected cattle LSDV was demonstrated in saliva at least for 11 days after the development of fever, in semen for 42 days and in skin nodules for 39 days. Viraemia occurs after the initial febrile reaction which persists for two weeks. Viral replication in pericytes, endothelial cells and probably other cells in blood vessel and lymph vessel walls causes vasculitis and lymphagitis in some vessels in affected areas (El-Bagoury, 2012). Immunity after recovery from natural infection is life-long in most cattle; calves of immune cows acquire maternal antibody and are resistant to clinical disease for about six months (Coetzer, 2004). The clinical signs of the disease include fever, skin and mucous membrane nodules, and enlarged lymph nodes (Davies, 1991; Hunter & Wallace, 2001)

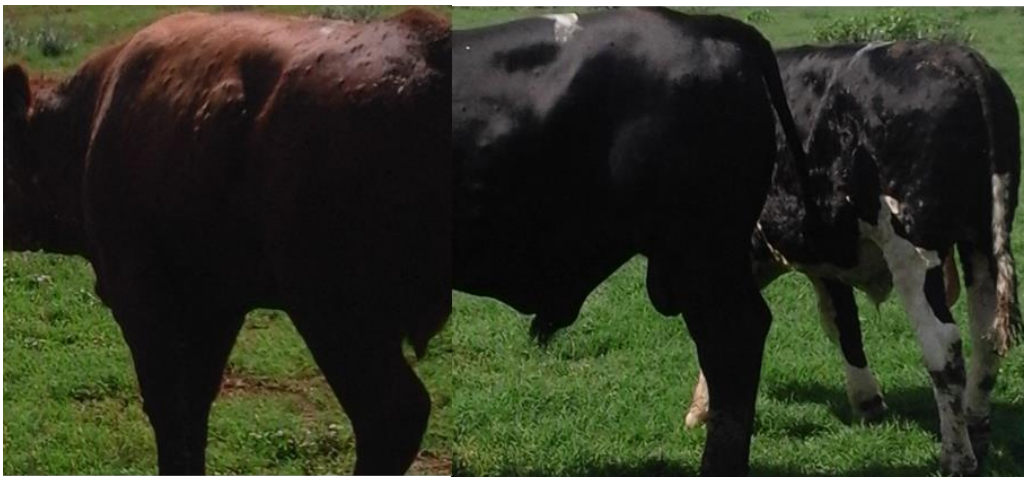


Figure 2.3: Characteristic skin lesions in local feedlot cattle in South Africa infected with Lumpy skin disease virus. Sources: [Abutarbush *et al*., (2015); Babiuk *et al*., (2008)]

2.5.2.2 Geographic Distribution of LSDV

The first outbreak of LSDV was in 1929 in Zambia (Tuppurainen & Oura, 2012). Since the first outbreak, the disease has spread throughout the African continent, including Madagascar (Tuppurainen & Oura, 2012; Abutarbush *et al*., 2015). Libya, Algeria, Morocco, and Tunisia are the only African countries which are still considered to be free of LSDV (Coetzer, 2004). In May 1988, Egypt had its first outbreak of LSDV, with no trace of the origin of the outbreak (Tuppurainen & Oura, 2012; Abutarbush *et al*. 2015) and it was suspected that transmission was by *S. calcitrans* (Tuppurainen & Oura 2012). According to the OIE (2008), LSDV outbreaks have been reported in the Middle Eastern countries since 1990 including Kuwait, Lebanon, Yemen, United Arab Emirates, Bahrain, Israel and Oman (Tuppurainen & Oura, 2012).

This study seeks to determine whether the feedlot stable flies in South Africa are harbouring LSDV.

2.5.3 Ehrlichiosis

Ehrlichiosis are tick-borne diseases caused by small, pleomorphic, gram negative, obligate intracellular bacteria in the genus *Ehrlichia* which is closely related to genus *Anaplasma* belonging to the family Anaplasmataceae, and order Rickettsiales (Rikihisa, 1991; McQuiston *et al*., 2003; Bremer *et al*., 2005; Lee *et al*., 2005). The erhlichial pathogens are widespread in nature and are classified as α-proteobacteria. Their reservoir hosts include animals as well as human (Lee *et al*., 2005). Currently, the genus *Ehrlichia* contains five recognized species: *E. ruminantium*, *E. ewingii, E. chaffeensis*, *E. muris* and *E. canis* (Wen *et al*., 2002; Rikihisa, 1991). *E. canis* causes canine monocytic ehrlichiosis (CME) which is rarely implicated in human illness. *E. chaffeensis* also infects monocytes, causing illness in both dogs and people. In humans, the disease is called human monocytic ehrlichiosis (HME) (Rikihisa, 1999) and has been reported in more than 30 states in USA, Europe, Africa, Middle East, and Asia (Lee *et al*., 2005). The *E*. *ewingii* infects granulocytes and is zoonotic. It is sometimes known as canine granulocytic ehrlichiosis in dogs (Ganguly & Mukhopadhayay, 2008). However, human ewingii ehrlichiosis (HEE) is now the preferred name for the disease in humans. The *E. ruminantium* (formerly *Cowdria ruminantium*) is the agent of heartwater

in ruminants (Nakao, 2010) and is thought to be zoonotic following its detection in several infected people in South Africa (Nakao, 2010). For many years, *Ehrlichia* species have been known to cause illness in pets and livestock. The consequences of exposure vary from asymptomatic infections to severe, potentially fatal illness.

2.5.3.1 Geographic distribution of ehrlichiosis

E. canis was first reported in Algeria in 1935, and now has a worldwide distribution which varies with the presence and density of their tick vectors (Wen *et al*., 1997). The *E. chaffeensis* was originally described from North America, but it was recently detected in parts of South America, Asia and Africa (Ganguly & Mukhopadhayay, 2008). It is possible that some of these reports involve other closely-related organisms. Within the U.S., *E. chaffeensis* infections occur mainly in the southeastern, south-central and Mid-Atlantic States, where its major tick vector (*Amblyomma americanum*) is endemic (Ganguly & Mukhopadhayay, 2008). *E. ewingii* is also transmitted by *A. americanum* in North America, and it has been found in deer, other animals and ticks throughout this tick's range.

2.5.3.2 Pathogenesis

The ehrlichial pathogen can cause disease only at higher dosages. Although *Ehrlichia* spp. can be isolated from blood at the acute stages of infection, each *Ehrlichia* species seems to have a characteristic tissue tropism that causes a site-specific disease. *E. risticii*-infected cells are found predominantly along the intestinal wall, especially in the equine large colon (Rikihisa *et al*.,1985), where they cause watery diarrhea. *E. canis*-infected cells are commonly found in the microvasculature of the canine lungs, kidneys, and meninges (Huxsoll *et al*., 1972; Hildebrandt *et al*.,1973; Simpson, 1974). Epistaxis is caused by characteristic hemorrhages in the lungs or nasal mucosa. *E. sennetsu* (Misao & Kobayashi, 1954) and *Neorickettsiae* spp. (Frank *et al*.,1974) are predominantly localized in lymph nodes, where they cause severe lymphadenopathy. The *E. ruminantium* commonly localizes in the endothelial cells of the brain tissue (Cowdry, 1925), where it causes severe neurologic signs. Generally, patients with ehrlichiosis display remarkable lesions such as cell lysis, tissue necrosis, abscess formation, or severe inflammatory reactions, especially in the acute stages of the

disease. Thrombosis, endothelial-cell hypertrophy/hyperplasia and vasculitis with leukocyte infiltration around blood vessels, all of which generally occur during diseases caused by the rickettsiae, are usually absent during acute ehrlichial infection. Non-follicular lymphadenopathy is frequently seen in ehrlichial infection. Disappearance of follicles, small lymphocyte depletion and histiocytosis in local lymph nodes are features commonly noted during infection by *E. risticii*, *E. sennetsu*, *E. canis*, *E. phagocytophila*, and *N. helminthoeca* (Hudson, 1950; Ohtaki & Shishido, 1965; Hildebrandt *et al*., 1973; Frank *et al*., 1974; Harvey *et al*., 1979; Rikihisa *et al*.,1987; Rikihisa *et al*., 1988). In *E. platys* and *N. elokominica* infection, follicles in the lymph nodes remain active (Frank *et al.,* 1974; Baker *et al*.,1987).

In this study, the vector-pathogen relationship between South African feedlot stable flies and *Ehrlichia* spp. will be investigated.

2.5.4 Rickettsiosis

Rickettsioses is caused by obligate-intracellular gram-negative bacteria of the genus *Rickettsia,* belonging to the family Rickettsiaceae, Order Rickettsiales (Parola *et al*., 2003; Ndip *et al*., 2004). They are now recognized as important emerging vector-borne human infections worldwide (Parola *et al*., 2003). Many species of this genus are vertically transmitted symbionts of invertebrates (Yssouf *et al*., 2014) suggesting that transmission to animals and humans occurs via arthropod vectors including ticks, mites, lice, and biting flies with many of these arthropod vectors serving as reservoirs or amplifiers of rickettsiae (Yssouf *et al*., 2014; Kuo *et al*., 2015). Eight tick-borne rickettsioses with distinct species as agents have definitively been described throughout the world, including *Rickettsia rickettsii* (in the America), *R. sibirica* (in Asia), *R. conorii* including different strains (in Europe, Asia, and Africa), *R. australis* (in Australia), *R. honei* (in the Flinders Island, Australia), *R. japonica* (in Japan), *R. africae* (in sub-Saharan Africa and the West Indies), and *R. slovaca* (in Europe).

2.5.4.1 Geographic distribution of ricketssiosis

The etiological agent of rocky mountain spotted fever (RMSF) is *R. rickettsii*, which occurs primarily in the United States and is transmitted to humans by Ixodid tick species (Tzianabos *et al*., 1989), whereas *R*. *conorii* is the causative agent of human tick-bite

fever found in Southern Africa and the Mediterranean, is transmitted by the brown dog tick *Rhipicephalus sanguineus* (Shpynov *et al*., 2015). The *R. japonica* and *R. australis* are widely distributed in Asia and Australia and infect man through various species of animal ticks. The *R. tsutsugamushi* was recently renamed as a new genus with only one species *Orientia tsutsugamushi*. It is the agent of scrub typhus acquired from the bite of larval trombiculid mites living on the waist high *Imperata* grass growing in previously cleared jungle around villages and in plantations. The causative agent of African Tick Bite Fever (ATBF), *R. africae*, is found in the African veld and is transmitted in game park areas by ticks living on cattle, hippo, and rhino (Althaus *et al*., 2010). In the sub-Saharan region, *R. africae* is transmitted by tick species *Amblyomma variegatum,* with *Amblyomma hebraeum* transmitting *R. africae* in South Africa (Althaus *et al*., 2010; Shpynov *et al*., 2015). In southern Zimbabwe, an endemic area for *R. africae* infection, almost 100% of cattle were found to have antibodies to Spotted fever group (SFG) rickettsiae (Parola, 2004).

2.5.4.2 Pathogenesis

Ricketssial organisms develop in the alimentary canal of arthropods (Rathi & Rathi, 2010). The arthropods maintain the infection naturally by either transovarial transmission by acting as a vector and a reservoir or without the transovarial transmission where the arthropod only acts as a vector (Rathi & Rathi, 2010). The clinical symptoms of spotted fever group rickettsioses generally begin 6–10 days after the arthropod bite and typically include fever, headache, muscle pain, rash, local lymphadenopathy, and a characteristic inoculation eschar associated with a few vesicular lesions (Raoult *et al*., 2001). However, the main clinical signs vary depending on the rickettsial species involved and may allow for distinction between several SFG rickettsiosis occurring in the same location. For example, ATBF is characterized by the high frequency of multiple inoculation eschar at the tick- or flea-bite site (Parola *et al*., 2005) a typical sign of spotted fever group rickettsioses. This is because numerous highly infected *Amblyomma* ticks may attack and bite many people in several places at the same time whereas in the case of Mediterranean spotted fever due to *R. conorii*, a single eschar is usually due to the low affinity of the tick to bite people and a low rate of infection of the ticks (Rathi & Rathi 2010). This study seeks to investigate whether there is a relationship between feedlot stable flies and *Rickettsia* spp. in South Africa.

2.6 Metagenomics

The discipline of metagenomics is defined as culture-independent genomic analysis of all the small-scale organisms in a specific environmental niche (Handelsman *et al*., 1998). It has evolved in an effort to discover more about the microbial diversity of natural environments, such as soil, marine water and the gastro intestinal tracts of vertebrates and invertebrate communities (Lo'pez-Garcı'a & Moreira, 2008). Metagenomics is another and increasingly sophisticated field which in its least difficult terms is concerned with the isolation of DNA from a defined habitat, trailed by cloning of the complete genomes of the entire microbial population in their natural surroundings (Langer *et al*., 2006).

The resulting DNA is then analyzed for functions and sequences of interest. Metagenomics can be divided into sequence-based and function-driven analysis of uncultured micro-organisms (Gabor *et al*., 2007).

Functional metagenomics involves screening metagenomics libraries for a particular phenotype, e.g. salt tolerance, antibiotic production or enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Dinsdale *et al*., 2008). Sequence-based approaches, on the other hand, involve screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the entire clone to identify other genes of interest, or large-scale sequencing of the complete metagenome to search for phylogenetic anchors in the reconstructed genomes (Riesenfeld *et al*., 2004; Hoff e*t al*., 2008).

What sets next-generation sequencers apart from conventional capillary-based sequencing is their ability to process millions of sequence reads in parallel rather than 96 at a time, it can be used if a *de novo* genome sequence is to be assembled from the next-generation data, and also produces shorter read lengths (35–300 bp, depending on the platform) than capillary sequencers (650–800 bp), which can also impact the utility of the data for various applications such as *de novo* assembly and genome resequencing (Mardis, 2008).

2.6.1 Insect gut microbial communities

Over the past decades, there has been an increase in studies on insect's gut microbes to better understand the role they play in the metabolism of insects (Engel & Moran, 2013). A few metagenomics studies have been conducted looking into the hindgut and midgut of the wood-feeding 'higher' termite and the gypsy moth (*Lymantria dispar*) respectively (Warnecke *et al*., 2007). Termites are widely known as ecologically essential wood-degrading organisms (Sugimoto *et al*., 2000) with crucial ecological roles in the turnover of carbon and additionally serving as prospective source of biochemical catalyst which can be used in the conversion of wood to biofuels (Warnecke *et al*., 2007). Significant data has recently emerged which suggests that the symbiotic bacteria resident in the hindgut of the termite play a functional role in the hydrolysis of both cellulose and Xylan (Tokuda & Watanabe, 2007). Similarly, studies on the bacterial communities associated with *Ixodes Ricinus* ticks revealed the detection of pathogenic and non-pathogenic bacteria such as *Borrelia*, *Rickettsia*, and *Candidatus* Neoerhlichia (Carpi *et al*., 2011), and those with mutualistic relations such as *Wolbachia* and Rickettsiella. Furthermore, ecological analysis on the study revealed that the bacterial community structure differed between the examined geographic regions and tick life stages (Carpi *et al*., 2011). Previous studies have been conducted for detection and analysis of gut microbes from economically important insects such as fruit flies, wood mites, cockroaches, earwigs, honey bees, moths, weevils, bristletails, and stinkbugs to have a better understanding of their symbiotic relations (Sleator *et al*.,2008; Sabree & Moran, 2014; Yun *et al*.,2014).

This study has attempted to detect microbiota harboured by *S. calcitrans* occurring in South African feedlots using *16S rRNA* metabarcoding using the Illumina next generation sequencing platform.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study areas

The fly samples for the study were collected at three provinces in South Africa, namely North West, Free State, and Limpopo (Figure 3.1). The overall landscape size, seasonal climatic conditions as well as vegetation in the three sampled provinces varies significantly and as a result the abundance, distribution and species diversity of *Stomoxys* is considerably different.

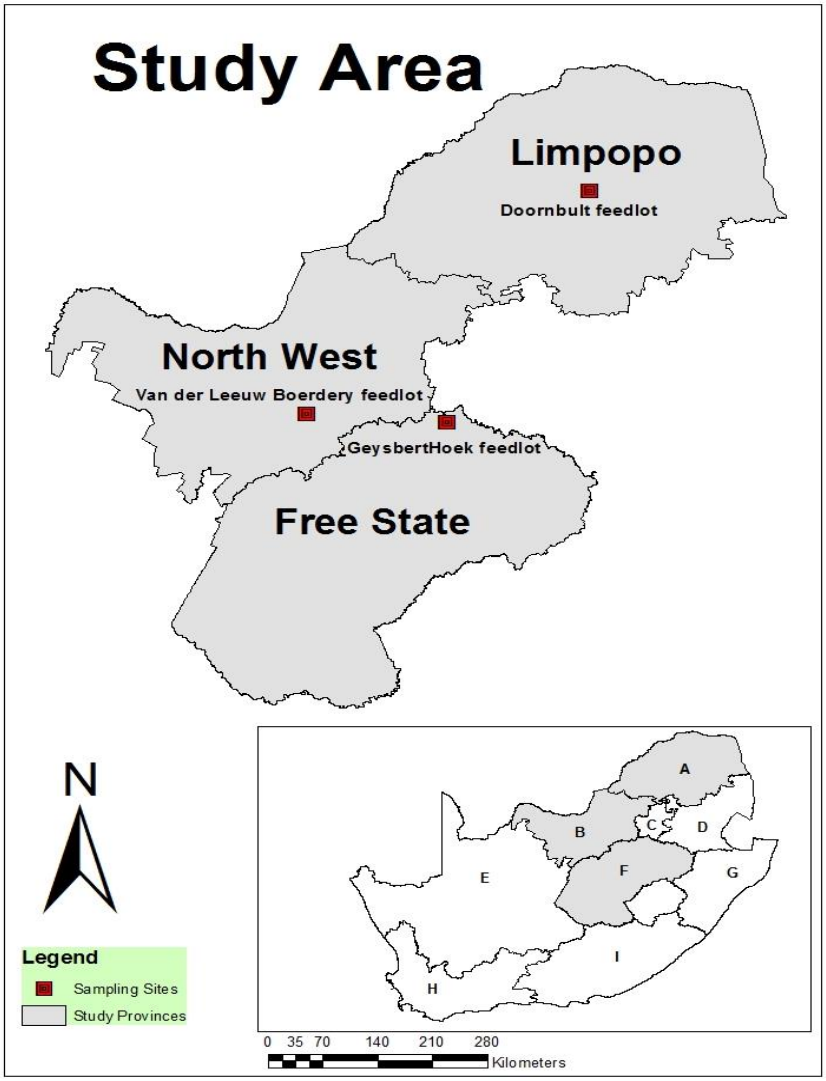


Figure 3.1: Map of South Africa showing the three sampled provinces, namely, Limpopo **(A)** North-West **(B),** and Free State **(F)** which are all indicated by boxes.

3.1.1 Van der Leeuw Boerdery feedlot in North West Province

The fly specimens were collected at Van der Leeuw Boerdery feedlot (26°52'41.1" S; 27°00'21.8" E) situated near Potchefstroom, JB Marks local municipality, North West Province (Figure 3.2). The number of cattle accommodated by this feedlot is unknown. The North West Province is an inland South African province that borders Botswana. Its landscape is defined by mountains in the northeast and bushveld scattered with trees and shrubs. The minimum and maximum temperatures range from 17°C to 31 °C in the summer and from 3°C to 21 °C in the winter. Annual precipitation totals about 360 mm, with almost all of it falling during the summer months, between October and April (www.south-africa-info.co.za)

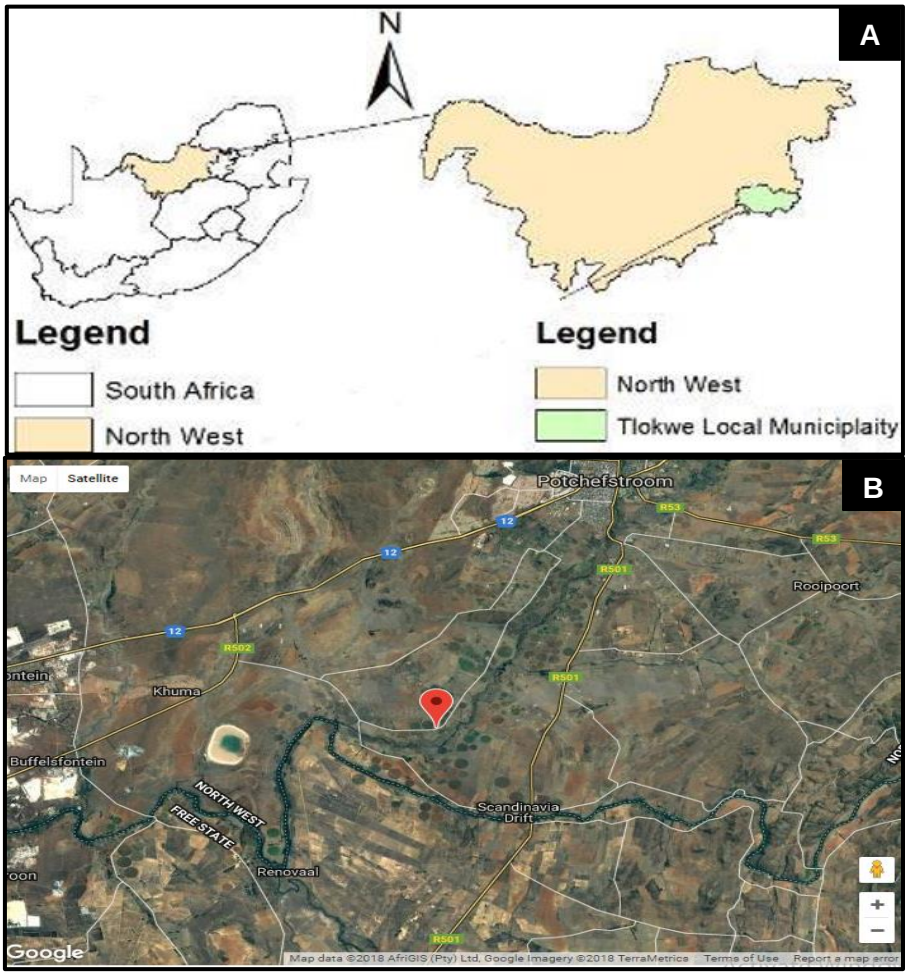


Figure 3.2: Map of South Africa showing: **(A)** North West Province and the JB Marks local municipality. **(B)** The Google Earth image of the location of Vander Leeuw Boerdery feedlot (www.google.co.za).

3.1.2 GysbertHoek feedlot Free State Province

The fly specimens were collected at GysbertHoek feedlot near Sasolburg, Metsimaholo Local Municipality, (26°54' 36.0 S; 27°48'59" E), Free State Province (Figure 3.3). The feedlot can accommodate approximately 13 000 head of cattle. The Free State Province is a South African province sprawling over high plains and stretching along the Maluti Mountains bordering Lesotho. The minimum and maximum temperatures range from 13 °C to 31 °C in summer and from min: -2 °to 17 °C in winter, with an annual precipitation of 680 mm (www.south-africa-info.co.za).

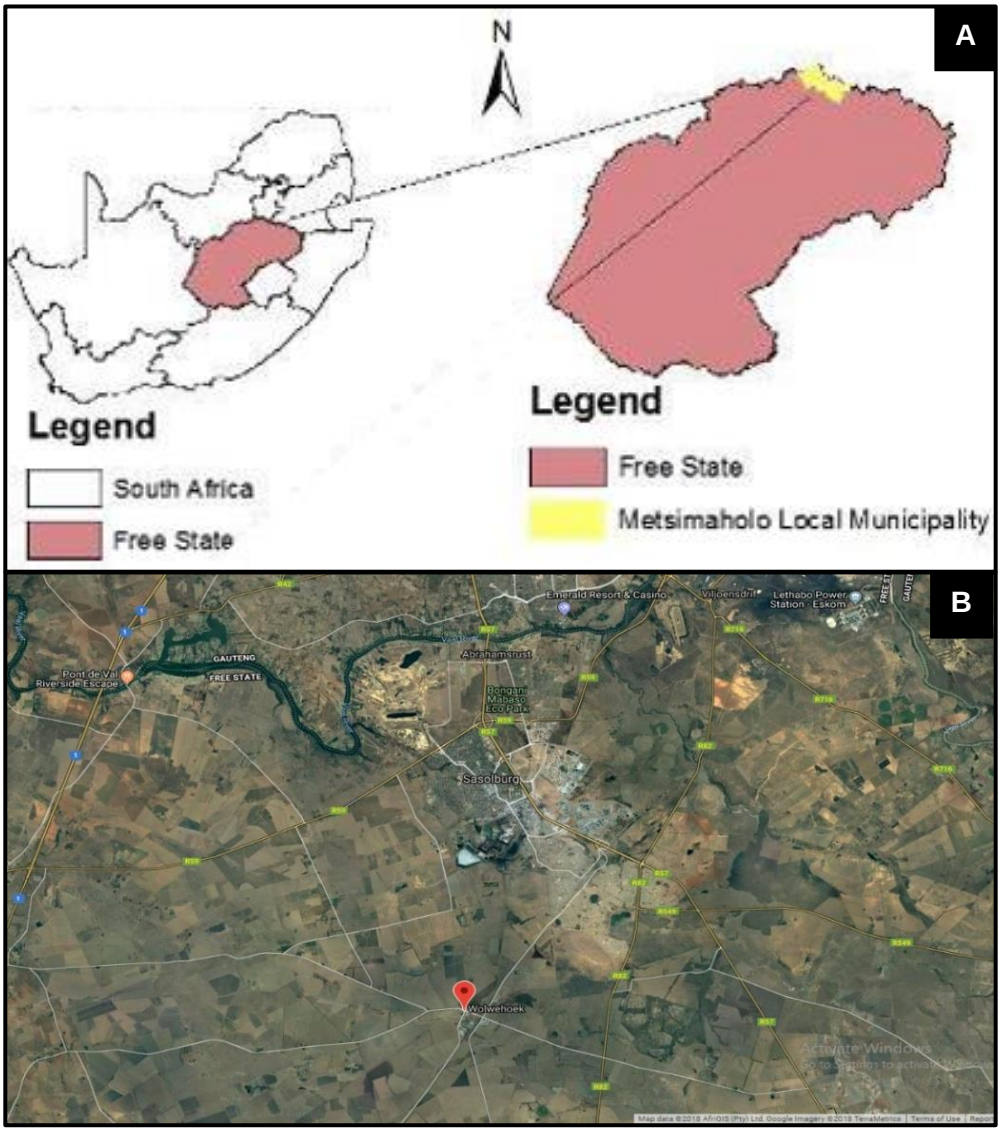


Figure 3.3: Map of South Africa showing: **(A)** Free State Province and the Metsimaholo local municipality. **(B)** The Google Earth image of the location of GysbertHoek feedlot (www.google.co.za).

3.1.3 Doornbult feedlot in Limpopo Province

The fly samples were collected at Doornbult feedlot near Polokwane City, Polokwane Local Municipality (23°47'42.914" S; 29°25'17.155") Limpopo Province (Figure 3.4). The feedlot occupies an area 0.21km^2 and can accommodate approximately 5000 head of cattle. Limpopo Province is a South African province bordering Botswana, Zimbabwe and Mozambique. It occupies an area of 125 754 km² and is characterized by its bushveld and wildlife reserves. Temperature ranges with an average of 23°C in summer and 13°C in winter, with an average annual rainfall of 100mm (www.south-africa-info.co.za).

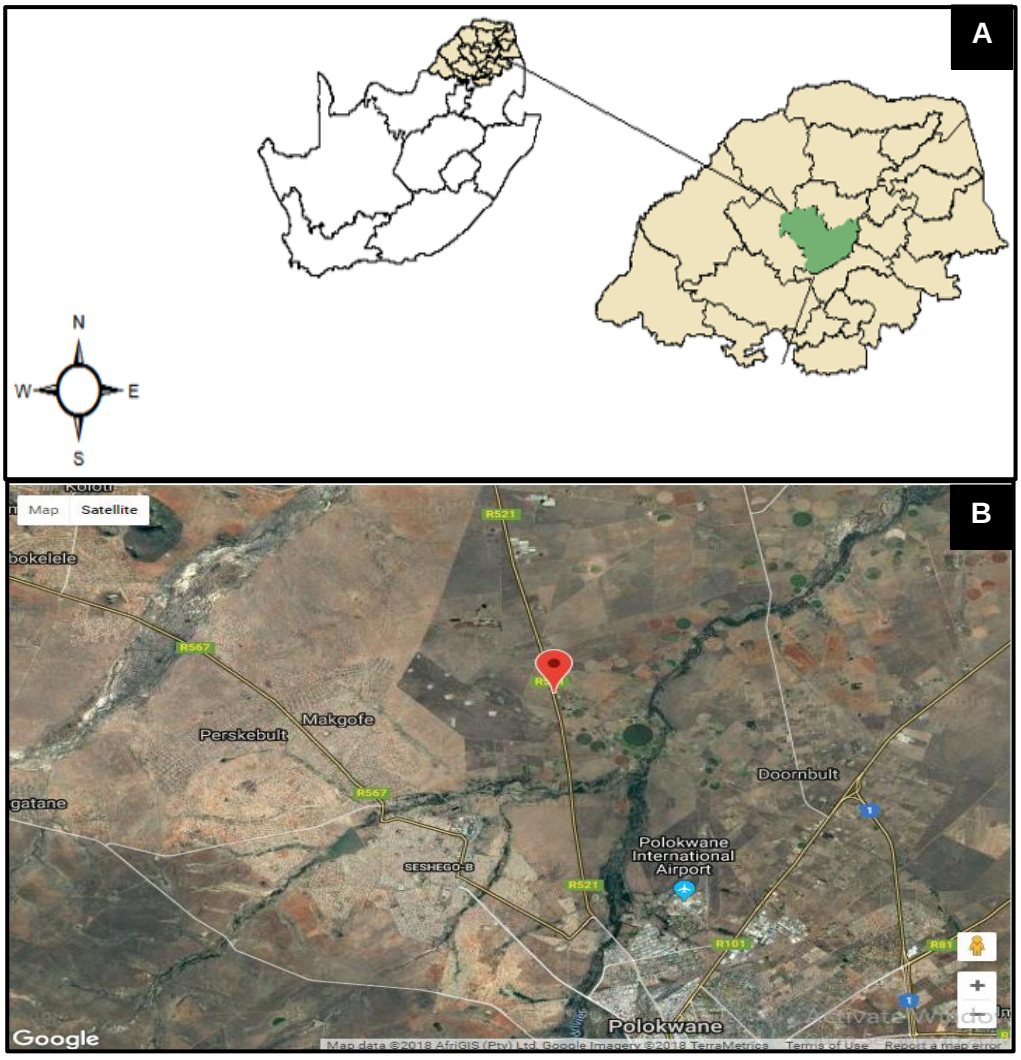


Figure 3.4: Map of South Africa showing: **(A)** Limpopo Province and Polokwane local municipality.**(B)** The Google Earth image of the location of Doornbult feedlot (www.google.co.za).

3.2 Collection of stable flies

The flies were captured using Vavoua traps (Plate 3.1) that were placed at three study areas as feedlots. A total of 9 traps were set in three areas: 3 Vavoua traps at Geysbert Hoek feedlot, 3 Vavoua traps at Van der Leeuw feedlot and 3 traps set at Doornbult feedlot respectively. The Vavoua traps were monitored and changed in two day intervals to collect the captured flies. A total of 10195 stable files were collected from the traps and a total of 1570 by-catches were also collected from the traps. Upon collection in each trap, the flies were submerged in 70% ethanol and taken to the North West University laboratory for further analysis. Upon arrival at the University laboratory, the flies were identified under light microscope according to their morphology and recorded, separating stable flies from by-catches.

3.3 Identification and characterization of stable flies

The collected flies were identified using Wild Heerbrugg M5 stereo microscope (Heerbrugg, Switzerland) with reference to literatures of Zumpt (1973), Masmeatathip *et al*. (2006) and Dodge (1966). The Nikon AZ 100 M zoom microscope (Nikon Inc., Tokyo, Japan) was used to capture sequential photographs of different features of the flies. For stable flies collected, identification keys were used to confirm the occurrence as there is no documented literature about the occurrence and distribution of stable flies in South Africa.

Plate 3.1: Vavoua traps used to capture stable flies. The traps were not baited with any odour to attract flies. Source: Picture taken during sampling by Makhahlela (2017).

3.4. Molecular identification of flies

3.4.1 Method of washing

Three different washing procedures were investigated on stable flies (*S. calcitrans*) to determine which method was more effective for the removal of surface microbes. Prior to DNA extraction the flies were washed with 70% ethanol, 10% bleach, and 10% tween20. The fly samples were submerged in 70% ethanol for a period of 1 hour, 2 hours, and 3 hours respectively and rinsed with double distilled water. The same was done for 10% bleach and 10% tween20. The flies were then removed from the solutions, rinsed with double distilled water and left to dry on the bench before they were transferred to 1.5 ml Eppendorf tubes.

To determine the effectiveness of the washing methods, the flies were visualized under scanning electron microscopy (SEM) to determine whether surface bacteria was removed. For sample preparation, samples were transferred to 99.6% ethanol and left overnight to remove excess water, this was done twice. Samples were then placed in holders with perforations in 99.6% ethanol. Using liquid carbon dioxide (CO_2) the samples were critical point dried (Weibel & Ober, 2003). Under high pressure, the 99.6% ethanol was substituted with CO_2. Samples were transferred to a stub containing double sided-conductive tape and the stub was placed inside an IB2 Ion Coater (EIKO Engineering Co. Ltd., Ibaraki, Japan) and coated with a layer of gold.

3.4.2 Isolation of fly DNA

Genomic DNA was extracted from 500 flies per feedlot with a total of 100 pools from each feedlot, all the flies were morphologically identified and grouped according to feedlots and date of collection. The wings and legs were removed in order to avoid excess exoskeletons that may influence the enzymatic action of the ensuing response steps (Mekata *et al*., 2008). Thereafter, the flies were pooled (five flies per pool) to increase the DNA yield and then crushed into sterile 1.5 ml tubes. DNA was extracted from whole flies using salting-out method described by Nasiri *et al*. (2005). Eppendorf tubes containing the crushed flies were filled with 410 µl of DNA extraction buffer [(10 Mm Tris-HCl, pH 8.5?), 10 Mm EDTA and 1% sodium dodecyl sulphate (SDS)]. Eighty microliters of 10% SDS was added along with 10 µl of

proteinase K (Pro-K). Samples were then incubated for one hour at 56˚C for lysis and digestion after finger vortexing the contents. After an hour, 10 µl of proteinase K was added again to the mixture which was then incubated overnight at the same temperature to complete digestion. On the next day, the samples were centrifuged at 12000 rpm for 5 minutes. The supernatant was transferred to the second batch of temporary sterile 1.5 ml Eppendorf tubes, adding 510 µl of the supernatant to each tube. One hundred and eighty microliters of 5 M NaCl was added to each tube containing the supernatant and mixed by vortexing vigorously for 30 seconds, followed by centrifuging at full speed (15 000 rpm) for 5 minutes. The supernatant was then transferred to the third and final batch of sterile 1.5 ml Eppendorf tubes. Four hundred and twenty microliters of ice-cold isopropanol (Propan-2-ol) were added to each tube and the contents were mixed by inverting the tubes 50 times. The contents were centrifuged at full speed for 15 minutes at 4˚C to precipitate the extracted DNA. The supernatant was discarded, and the isopropanol was removed by swirling the tube slowly. Finally, the pellet was washed with 70% ethanol and finger vortexed and then spun for 5 minutes; this step was repeated twice. Samples were left to air dry on the bench for an hour at room temperature, covered with a lint-free lab tissue. DNA pellet was dissolved in 200 µl of double distilled water (ddH_20). A NanoDrop spectrophotometer (Thermo Fisher, USA) was used to confirm the presence and concentration of the DNA before storage at -20˚C until used.

3.4.3 Amplification of *CO1* and 1*6S rRNA* stable fly genes

To supplement morphological analysis in characterizing the captured flies, PCR targeting the *CO1* and *16S rRNA* genes was conducted to amplify *S. calcitrans* DNA resulting in 710 bp and 300 bp of *CO1* and *16S rRNA* PCR amplicons respectively. The reactions contained a final volume of 25 µl which was composed of 12.5 µl of Amplitaq Gold 360 PCR Master Mix (Amplitaq Gold DNA Polymerase 0.05 units/µl, Gold buffer [30 Mm Tris/HCl Ph 8.05, 100 Mm KCl], 400 mm each dNTP and 5 mm $MgCl_2$) (Applied Biosystems, California, USA). Then 1.5 µl of each primer (10 µM each), 2 µl of template DNA and nuclease-free water to adjust the final reaction volume. For the *CO1* gene, the PCR conditions were set as follows: initial denaturation at 95°C for 10 minutes, followed

By 35 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds and final elongation at 72°C for 1 minute. When the *16S rRNA* was used the PCR conditions were set as follows: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 30 seconds and final elongation at 72°C for 1 minute. The primer sequences for *CO1* and *16S rRNA* genes (Table 3.1) amplified 710 bp and 300bp respectively. PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light using trans-illuminator.

Table 3.1. The *COI* and *16S rRNA* primer sequences used for PCR and sequencing

Primers	Sequences (5'->3')	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	Vrijenhoek,1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
N1-J-12585	GGTCCCTTACGAATTTGAATATATCCT	Marques *et al*., 2003
LR-N-12866	ACATGATCTGAGTTCAAACCGG	

3.4.4 Purification of amplicons

Positive PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany), whereby a 1% agarose gel stained with ethidium bromide was prepared. Thereafter, 20 µl of PCR amplicons were resolved by gel electrophoresis at 100 volts for 30 minutes. Subsequently, the gels with DNA fragments were cut out with a clean sharp scalpel and the gel slices were placed into 1.5 ml Eppendorf tubes. Then 600 µl of QG buffer was added. To dissolve the sliced DNA fragments in QG buffer, the Eppendorf tubes containing the solution were incubated at 50°C for 10 minutes, with vortexing the tubes every 2-3 minutes. Then 200 µl of isopropanol was added to increase the DNA yield. The mixture was transferred into QIAquick spin column with a 2 ml collection tube. To bind DNA the spin columns were centrifuged for 1 minute at 13 000 rpm. The flow through in the collection tubes was

discarded and an additional 500 µl of QC buffer was added to the QIAquick column and centrifuged for 1 minute at 13 000 rpm. The purified DNA in the QIAquick was then washed with 750 µl of PE buffer that contained 99% absolute ethanol and centrifuged again at 13 000 rpm for 1 minute. The flow through was discarded and the QIAquick column centrifuged to remove excess ethanol in the PE buffer. The QIAspin columns were transferred into new clean 1.5 ml tubes and 50 µl of EB buffer was used to elute the DNA. The purified DNA samples were stored at -20°C until they were sent for sequencing.

3.4.5 Sequencing

Twenty micro liters of all positive purified PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa.

3.4.6 Phylogenetic Analysis

Retrieved gene sequences obtained from all positively tested amplicons were edited using BioEdit to remove any degenerate base pairs (Hall, 1999) and saved as FASTA format. To confirm sequences obtained from *CO1* and *16S rRNA* analysis, the nucleotide basic local alignment search tool (BLASTn) was used. Only gene sequences with 98% to 100% similarity match score were considered as significant. Gene sequences of species closely related to *Stomoxys* flies in the current study from the BLASTn search results were downloaded from NCBI database. The *CO1* and *16S rRNA sequences* were aligned by ClustalW using multiple alignment fast Fourier transform (MAFFT) program to conduct multiple and pair-wise sequence alignment (Katoh & Standley, 2013). Thereafter, the aligned sequences were trimmed using TrimAl version 1.4 to remove the uneven ends from the aligned sequences. The trimmed alignment was subsequently transferred to MEGA 7 for Maximum likelihood (ML). For ML analysis 10 000 bootstrap replicates were used.

3.5 Molecular identification of pathogens

3.5.1 PCR for detection of *Erhlichia* spp.*, Rickettsia* spp., *Anaplasma marginale*, and LSDV

DNA was extracted from whole flies using modified salting-out method described by Nasiri *et al*. (2005) as described in section 3.4.2.

3.5.1.1 Amplification of *A. marginale* DNA

Species specific primers msp1α-1733F (5‟-TGTGCTTATGGCAGACATTTCC-3‟), msp1α-2957R (5”-AAACCTTGTAGCCCCAACTTATCC-3”), and msp1α-3134R (5‟-TAACGGTCAAAACCTTTGCTTACC -3‟) which amplified 1090-1113 and 1267-1290 bp of the msp1α gene fragment were used (Lew *et al*. 2002). The nested format (using 1733F and 3134R in the first PCR) was only used when a single amplification (using 1733F and 2957R) was not successful. The genomic DNA of *A. marginale* obtained from Onderstepoort Biological Products (OBP) in Pretoria was used as a positive control and double distilled water (ddH_2O) was used as a negative control. The PCR reaction had the final volume of 25 µl which consisted of 12.5 µl AmpliTaq Gold® 360 PCR Master Mix [(AmpliTaq Gold® DNA Polymerase 0.05 units/ µl, Gold buffer [30 mM Tris/HCl pH 8.05, 100 mM KCl], 400 mM of each dNTP and 5 mM $MgCl_2$)] (Applied Biosystems, California, USA). Then 2.5 mM of each primer, 2 µl of template DNA and ddH_2O was added to make the final volume. PCR conditions included initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, initial elongation at 72°C for 1 minute and final elongation step at 72°C for 7 minutes. Apart from substituting 60°C as the annealing temperature, the same conditions were used for the nested amplification using 5 µl of first reaction in the second reaction (Lew *et al*., 2002).

3.5.1.2 Amplification of *Ehrlichia* spp. DNA

The hypervariable V1 region of the *16S rRNA* gene was amplified using the primer pair 16S8FE (5′-GGAATTCAGAGTTGGATCATGGCTCAG-3′) and B-GA1B (5′-CGGGATCCCGAG TTTGCCGGGACTTCTTCT-3′) amplifying 476-bp fragment were used (Aktas *et al*., 2009). *Ehrlichia* positive DNA controls were obtained from the Center for Zoonosis Control (Hokkaido University, Japan) and double distilled water

(ddH_2O) was used as a negative control. The PCR reaction had the final volume of 25 µl which consisted of 12.5 µl AmpliTaq Gold® 360 PCR Master Mix [(AmpliTaq Gold® DNA Polymerase 0.05 units/ µl, Gold buffer [30 mM Tris/HCl pH 8.05, 100 mM KCl], 400 mM of each dNTP and 5 mM MgCl2)] (Applied Biosystems, California, USA). Then 2.5 mM of each primer, 2 µl of template DNA and ddH_2O were added to make the final volume of 25 µl. PCR conditions included initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, initial elongation at 72°C for 1 minute and final elongation step at 72°C for 7 minutes (Aktas *et al*., 2009).

3.5.1.3 Amplification of *Rickettsia* spp. DNA

The genus-specific primers CS-78 (5”-GCAAGTATCGGTGAGGATGTAAT-3”) and CS-323 (5”-GCTTCCTTAAAATTCAATAAATCAGGAT-3”) were used to amplify approximately 407 bp fragment of the *gltA* gene (Labruna *et al*., 2004). *R. africae* DNA obtained from the Center for Zoonosis Control (Hokkaido University, Japan) was used as positive control whereas ddH_2O was used as a negative control. The PCR reaction had the final volume of 25 µl which consisted of 12.5 µl AmpliTaq Gold® 360 PCR Master Mix [(AmpliTaq Gold® DNA Polymerase 0.05 units/ µl, Gold buffer [30 mM Tris/HCl pH 8.05, 100 mM KCl], 400 mM of each dNTP and 5 mM MgCl2)] (Applied Biosystems, California, USA). Then 2.5 mM of each primer, 2 µl of template DNA and ddH_2O were added to make the final volume of 25 µl. PCR conditions included, initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds, initial elongation at 72°C for 1 minute and final elongation step at 72°C for 7 minutes (Labruna *et al*., 2004).

3.5.1.4 Amplification of LSDV DNA

Primer pairs lsd43U (5”-GTGGAAGCCAATTAAGTAGA-3”), lsd383U (5”-CCCAATATTCTGCTGCTCTT-3”) and lsd1262L (5”-GTAAGAGGGACATTAGTTCT-3”) were used for the amplification of the 1237 bp fragment of LSDV NI-2490 genome (Stram *et al*.,2008). Double distilled water (ddH_2O) was used as a negative control. The PCR reaction had the final volume of 25 µl which consisted of 12.5 µl AmpliTaq Gold® 360 PCR Master Mix [(AmpliTaq Gold® DNA Polymerase 0.05 units/ µl, Gold buffer [30 mM Tris/HCl pH 8.05, 100 mM KCl], 400 mM of each dNTP

and 5 mM MgCl2)] (Applied Biosystems, California, USA). Then 2.5 mM of each primer, 2 µl of template DNA and ddH_2O were added to make the final volume. When the lsd43U and lsd1262L pair was used, the reaction conditions were: initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, initial elongation at 72°C for 1 minute and final elongation step at 72°C for 7 minutes. When the lsd383U and lsd1263 pair was used, the reaction conditions were: initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 49°C for 30 seconds, initial elongation at 72°C for 1 minute and final elongation step at 72°C for 7 minutes (Stram *et al*., 2008).

3.6 The *16S rRNA* library preparation workflow

3.6.1 Initial amplicon PCR

The initial amplicon PCR was conducted to amplify the targeted DNA sample using specific primers with overhanging adapters. The PCR reaction contained 12.5 µl of 2X KAPA Hifi HotStart Readymix [composed of KAPA HiFi HotStart DNA Polymerase (1 U/µL), Fidelity Buffer (5x), GC Buffer (5X), high-quality dNTPs (10 Mm each) and $Mgcl_2$ (25 Mm)] (KAPA Biosystems, Massachusettss, USA). 5 µl of forward primer (1µM) = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW GCAG 3’ and reverse primer (1µM) = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGGACTACHVGGGTATCTAATCC 3’ targeting the V3 and V4 region of the 16S rRNA gene with amplicon of approximately ~ 460 bp and lastly 2.5 µl of microbial DNA. The reaction had a final volume of 25 µl in 96 well PCR plates (Illumina, 2013). The PCR conditions involved an initial one cycle done at 95°C for 3 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds. Thereafter, a final one cycle of extension was done at 72°C for 5 minutes followed by a hold temperature at 4°C pending further analysis. One microliter of the PCR product was run on Agilent Bioanalyzer DNA 1000 (Agilent Technologies, Waldbronn, Germany) to verify the size of DNA libraries. The 470 bp was verified using the V3 and V4 primer pairs from the protocol, the expected size on a Bioanalyzer trace after the amplicon PCR step was ~550 bp.

3.6.2 Initial PCR clean-up

The amplicon PCR plate was centrifuged at 3,500 rpm for one minute at 20°C. AMpure XP beads (Beckman Coulter) were equilibrated to room temperature and vortexed for 30 seconds to ensure that the beads were in a homogeneous mixture (Illumina, 2013). Twenty microlitres of AMPure XP beads were added to each well of the PCR amplicon in the plate using a multichannel pipette. The samples were incubated at room temperature for 5 minutes without shaking to allow DNA to bind to the beads. The plate was placed on a magnetic stand for 2 minutes. Subsequently, the supernatant was removed with a multichannel pipette and discarded. The amplicon PCR plate was washed twice with 200 µl of freshly prepared 80% ethanol then incubated for 30 sec on the magnetic stand and the supernatant was discarded as before. A P20 multichannel pipette was used to remove excess ethanol. Subsequently, the amplicon PCR plate was air dried at room temperature for 10 minutes, thereafter, the amplicon PCR plate was removed from the magnetic stand and 52,5 µl of 10 Mm Tris, pH 8.5 was added to each amplicon. Thereafter, the amplicon was incubated at room temperature for 2 minutes then placed on a magnetic stand until the supernatant was dissolved. Then 50 µl of the supernatant transferred into a new 96-well plate.

3.6.3 Index PCR

A total of 5 µl of the PCR amplicons was transferred into a new 96-well plate and the remaining 45 µl was stored for further analysis. The index primers 1 and 2 were arranged according to the following order; index 2 primer tubes with orange caps were vertically aligned from A to H whereas, the index 1 primer tubes with orange caps were horizontally aligned from 1 to 12 in the TruSeq Index plate fixture (Illumina, 2013). Subsequently, the 96-well plate with the 5 µl re-suspended PCR amplicon was placed in the TruSeq index plate fixture. Subsequently, a 50 µl mixture containing 5 µl of Nextera XT index primer 1 (N7xx), 5 µl Nextera XT index primer 2 (S5xx), 25 µl of 2X KAPA HiFi HotStart ready Mix [composed of KAPA HotStart DNA polymerase (1 U/µL), Fidelity Buffer (5X), GC Buffer (5X), High quality dNTPs (10 Mm each) and $MgCl_2$ (25 Mm)] (KAPA Biosystem, Massachusetts, USA) and 10 µL of PCR grade water was prepared. The contents were mixed thoroughly, and the plate was covered with a Microseal ‘A’ then centrifuged at 3 500 rpm for 1 minute at

20°C. The PCR conditions were set as follows; 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation step of 72°C for 5 minutes with holding temperature at 4°C.

3.6.4 PCR clean-up 2

AMPure XP beads were used for this clean-up. The index PCR plate was centrifuged at 2 500 rpm at 20°C for 1 minute. As done before in the first clean-up, the AMPure XP beads were vortexed for 30 seconds to ensure that they are evenly dispersed (Illumina, 2013). A total of 56 µl of AMPure XP beads were added and thoroughly mixed into each well of the index PCR plate. The index PCR plate was then incubated at room temperature for 5 minutes. The index PCR plate was then placed on a magnetic stand for 2 minutes. Once again, a multichannel pipette was used to remove and discard the supernatant while the index PCR plate was on the magnetic stand. Subsequently, the beads were washed with 200 µl of freshly prepared 80% ethanol and incubated for 30 seconds on the magnetic stand. The supernatant was then removed and discarded. The 80% ethanol wash was repeated twice after which, the index PCR plate was air-dried for 10 minutes while on the magnetic stand. The index PCR plate was then removed and 27.5 µl of 10 mM Tris was added and mixed thoroughly into each well. The index PCR plate was subsequently incubated at room temperature for 2 minutes and then placed on a magnetic stand for 2 minutes. About 25 µl of the supernatant from the index PCR plate was transferred into a new 96-well plate, sealed with Micro-seal 'B' and stored at -25°C.

3.6.5 Library quantification, normalization and pooling

Firstly, DNA concentration was calculated and converted to nano molar (nM) as determined by an Agilent technology 2100 Bioanalyzer trace (Agilent Technologies, Woldbronn, Germany).

$$\frac{\text{Concentration in ng/µl}}{660\ \text{g/mol} \times \text{average library size}} \times 10^6 = \text{Concentration in nM}$$

The determined concentration was diluted with Tris-Cl buffer, pH 8.5 to reach a concentration of 4 nM for the library preparation (Illumina, 2013) from each library.

From each library, 5 µl of each barcoded DNA library (diluted to 2 nM) were pooled into single tube

3.6.6 Library denaturation and MiSeq sample loading

Heat block suitable for 1.7 ml microcentrifuge tubes was set to 96°C. MiSeq v3 reagent cartridge (Illumina, USA) was thawed at room temperature and ice water bath containing 3 parts of ice and 1 part of water was prepared (Illumina, 2013). Subsequently, 5 µl of freshly diluted 0.2 N NaOH was added to the 5 µl of 4 nM pooled library. Samples were briefly vortexed and centrifuged 2 500 rpm at 20°C for a minute and incubated at room temperature for 5 minutes to allow all DNA to denature into single strands. Finally, 990 µl of pre-chilled HT1 was added to 10 µl of the denatured DNA and the samples were placed on ice until final dilution stage.

3.6.7 Denaturation and dilution of phix control

During this part of the experiment, 2 µl of 10 nM PhiX library was mixed with 3 µl of 10 Mm Tris pH 8.5. Thereafter, 5 µl of 4 nM PhiX library and 5 µl of 0.2 N NaOH were mixed in a microcentrifuge tube and briefly vortexed. Then the samples were incubated for 1 minute at room temperature to denature the PhiX library to single strands. To produce 20 pM PhiX library, 10 µl of denatured PhiX library was added to 990 µl of pre-chilled HT1, the samples were then mixed by inverting several times, pulse centrifuged and placed on ice. Subsequently, the two libraries were combined by adding 30 µl of the denatured and diluted PhiX control with 570 µl of denatured and diluted amplicon library, and then placed on ice (Illumina, 2013). Using a heating block, the combined library and PhiX control were incubated at 96°C for 2 minutes, then inverted twice and placed on ice for 5 minutes. Samples were then loaded into MiSeq reagent kit V3 which targets the V3 and V4 region of the 16S rRNA gene with amplicon of approximately ~ 460 bp. cartridge (Illumina, USA) and analyzed using the MiSeq Reporter Software (MRS) using the metagenomic workflow option from the MiSeq system.

3.6.8 Metagenomic data analysis

3.6.8.1 Sequence preparation

Prior to metagenomic data analysis, VirtualBox 5.2.8 and QIIME 1.9.1 64 bit was installed. Additionally, before data analysis, PAired-end Assembler for Illumina sequences (PANDAseq), SILVA 128 reference database, usearch61, and FastQC version 0.11.7 were installed. Metagenomic sequences generated were recovered from the MiSeq system and retrieved in FASTQ format. FastQC version 0.11.7 (Schmieder & Edwards, 2011; Patel & Jain, 2012) was used to assess the quality of the individual sequences. Open source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010; Nguyen *et al.*, 2016) was used for metagenomic analysis.

3.6.8.2 Merging sequence reads

PANDAseq was used to merge the forward and reverse reads of each sample (Masella *et al.*, 2012; Cole *et al.*, 2014). Minimum and maximum length of the assembled reads were 250 and 475 bp, respectively. Minimum and maximum overlap between the forward and reverse reads were 75 and 200 bp, respectively. Sequences with a threshold of less than 0.7 were considered as low quality and were removed. All sequences containing uncalled nucleotides from unpaired regions were also removed. A Bayesian classifier was used to ensure all sequences were from prokaryotes.

3.6.8.3 Combining merged sequence labels

A mapping file was created consisting of a spreadsheet where the first four columns consisted of information of the sample name, barcode sequence, primer sequence, and the name of the merged sequences. Afterward, the mapping file was saved as a CSV tab delimited file. Mapping file was then validated, and necessary corrections were made. By using the mapping file all the individually merged sequences were combined into a single FNA file used for operational taxonomic unit (OTU) picking.

3.6.8.4 Operational taxonomic units (OTU) picking

For this part of metagenomic data analysis sequences with 97% or above similarity were clustered together into OTU's. Open-reference OTU picking was done where sequences were clustered using a reference database and non-matching sequences were clustered *de novo* (Bik *et al.*, 2012) Open-reference OTU picking was done by using SILVA 128 reference database (Quast *et al.*, 2013; Yilmaz *et al.*, 2013) and usearch61 (Edgar, 2010; Kopylova *et al.*, 2016).

3.6.8.5 *Alpha* and *Beta* diversity analysis

Alpha and Beta diversity analysis of metagenomic data were conducted and visualized using QIIME and Rstudio. Alpha diversity was calculated for samples by location using detected OTUs. Shannon entropy of counts, Simpson's index, and chao1 richness estimators were used to standardize the dataset. Results were rarefied to a sequencing depth of 1000 sequences. Beta diversity analysis was performed to determine similarity and differentiation between the members and the sampled location.

CHAPTER 4

RESULTS

4.1 Morphological analysis

A total of 10 195 stable files were collected from three sampled provinces (Table 4.1). Additionally, a summary representing other insects that were caught as by-catch is given in table 4.2. Identification of stable flies (*S. calcitrans*) fly species was based on their morphological characteristics with reference to previously published keys as follows:

- The adult stable fly is 4 to 7 mm in body length (Plate 4.1 A&D) (Gerry *et al*., 2007; Masmeatathip *et al*., 2006).

- The thorax has four dorsal longitudinal stripes of which the two furthest stripes are shorter (Plate 4.1A) (Howel *et al*., 1978; Masmeatathip *et al*., 2006).

- The adult stable fly has grey abdomen and thorax set apart with an arrangement of patterns (Masmeatathip *et al*., 2006). The stable fly can further be recognized from different *Stomoxys* species by their bowed fourth wing vein (Plate 4.1B) (Dodge, 1953; Masmeatathip *et al*., 2006) and maxillary palps which are shorter than the proboscis (Bishop, 1913).

- The abdomen has a checkered pattern, one middle spot and two parallel round spots, on the dorsal side of the second and third segment (Plate 4.1C) (Howel *et al*., 1978).

- The stable fly has a blade like proboscis (Plate 4.1 E&F) (Bishop, 1913; Masmeatathip *et al*., 2006).

- The proboscis is a long, thin puncturing organ that projects forward from under the head (Bishop, 1913). The base is furnished with sclerotized teeth adjusted for cutting, tearing and puncturing (Stephens & Newstead, 1907).

- Different qualities used to separate between Stomoxyine flies incorporate the male genitalia and the outer morphological characters, for example, the dorsal abdominal designs (Masmeatathip *et al*., 2006).

Table 4.1. The numbers of stable fly species captured by Vavoua-traps

Geysbert Hoek feedlot				Van der Leeuw boerdery feedlot				Doornbult feedlot			
Dates	**Trap no.**	***S. calcitrans***	**By-catch**	**Dates**	**Trap no.**	***S. calcitrans***	**By-catch**	**Dates**	**Trap no.**	***S. calcitrans***	**By-catch**
01/03/2017	1	32	149	02/03/2017	4	6	87	04/03/2018	1	2	45
01/03/2017	2	0	100	02//03/2017	5	174	51	15/03/2018	2	25	30
09/03/2017	3	53	126	10/03/2017	4	10	93				
04/04/2017	1	90	162	10/03/2017	5	441	112				
				16/03/2017	4	46	76				
				16/03/2017	5	5284	172				
				23/03/2017	5	2345	122				
				04/04/2017	5	1687	230				
Totals		**175**	**537**			**9 993**	**943**			**27**	**75**

Table 4.2. Insects captured as by-catch from all sampled feedlots

Province	Order	Family	Genus	Total number
North West	Diptera	Muscidae	*Musca*	358
	Diptera	Culicidae	*Culex*	287
			Anopheles	196
	Hymoneptra	Argidae	*Arge*	5
		Apidae	*Apis*	5
	Lepidoptera	Hepialidae	*Eudalaca*	6
		Nymphalidae	*Cynthia*	7
	Isoptera	Termitidae	*Odontotermes*	6
	Coleoptera	Coccinellidae	*Cheilomenes*	5
	Mantodea	Mantidae	*Sphodromantis*	7
			Compsothespis	9
Free State	Diptera	Muscidae	*Musca*	239

		Culicidae	*Culex*	187
			Anopheles	99
	Neuroptera	Chrysopidae	*Chrysemosa*	5
	Orthoptera	Acrididae	*Acrida*	7
Limpopo	Diptera	Muscidae	*Musca*	30
	Diptera	Culicidae	*Culex*	28
	Diptera	Oestridae		12
Total				**1555**

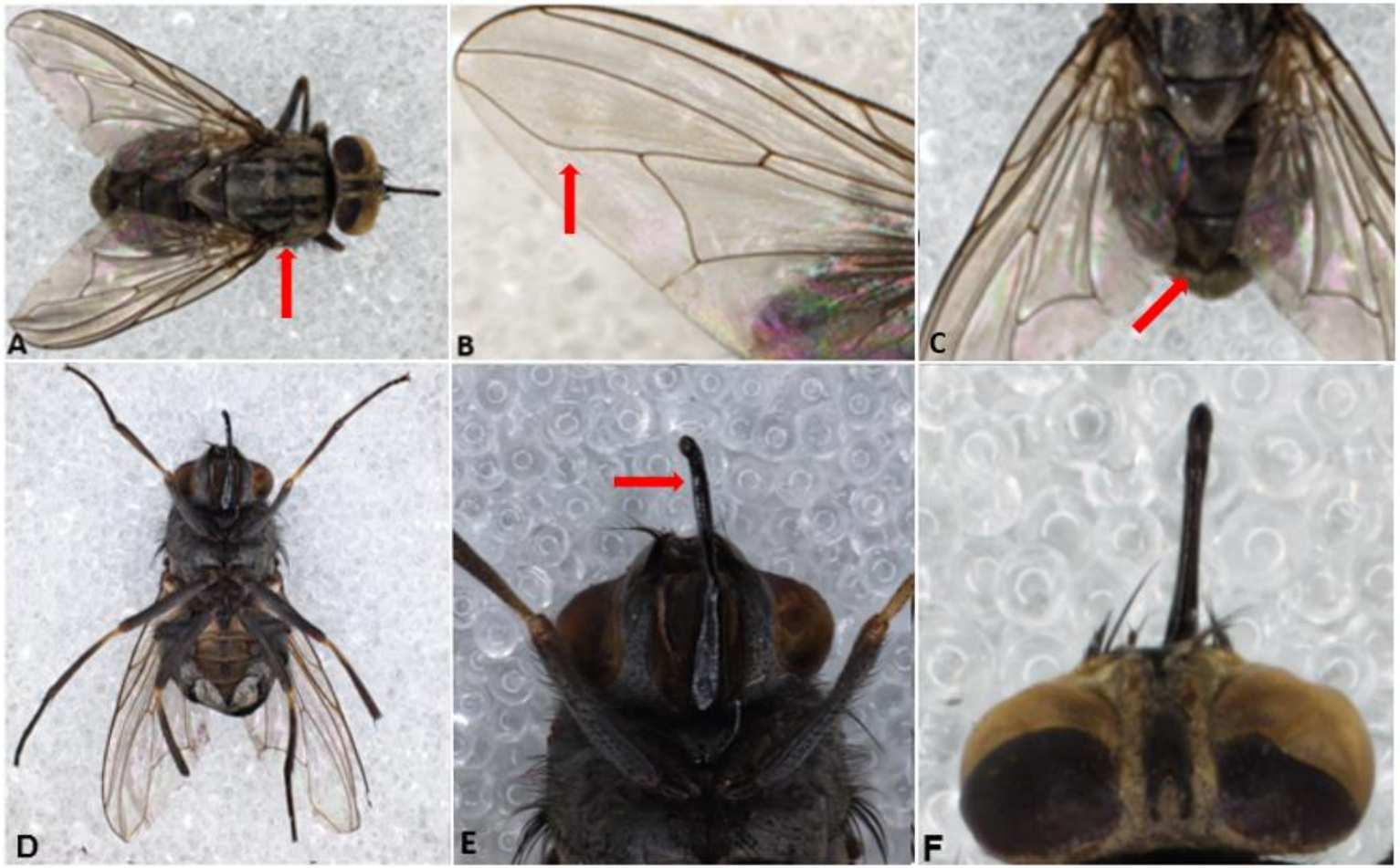


Plate 4.1: Morphological features of stable fly (*Stomoxys calcitrans*). **(A)** dorsal view with arrow showing checkered pattern on thorax; **(B)** wing with arrow indicating bowed fourth wing vein; **(C)** dorsal view of the abdomen with a checkered pattern; **(D)** ventral view of the whole fly; **(E)** ventral view of the Proboscis; **(F)** dorsal view of the head and proboscis

4.2 Sterilization of stable fly for removal of external microbes

The scanning electron microscope (SEM) results on treated (sterilized) flies showed clear differences between different sterilitization treatments. The flies sterilized with 10% bleach and 10% tween20 appeared to be smooth and clean of any external microbial flora (Plate 4.2 A to F), whereas, flies sterilized with 70% EtOH exhibited clusters of microbial flora on the external surface (Plate 4.2 G, H, and I)

4.2.1 PCR for assessement of fly DNA after sterilization of the flies

DNA was extracted from flies sterilized with tween20, 10% bleach and 70% ethanol. The presence of DNA was confirmed by agarose gel eletrophosesis as shown in figure 4.1. Furthermore, PCR was conducted to determine the qaulity of the DNA extracted from flies sterilized with tween20, 10% bleach and 70% ethanol was subjected to PCR in order to determine whether it is not damaged. All DNA of flies sterilized with 3 different reagents was amplified by PCR targeting the *16S rRNA* (Figure 4.2).

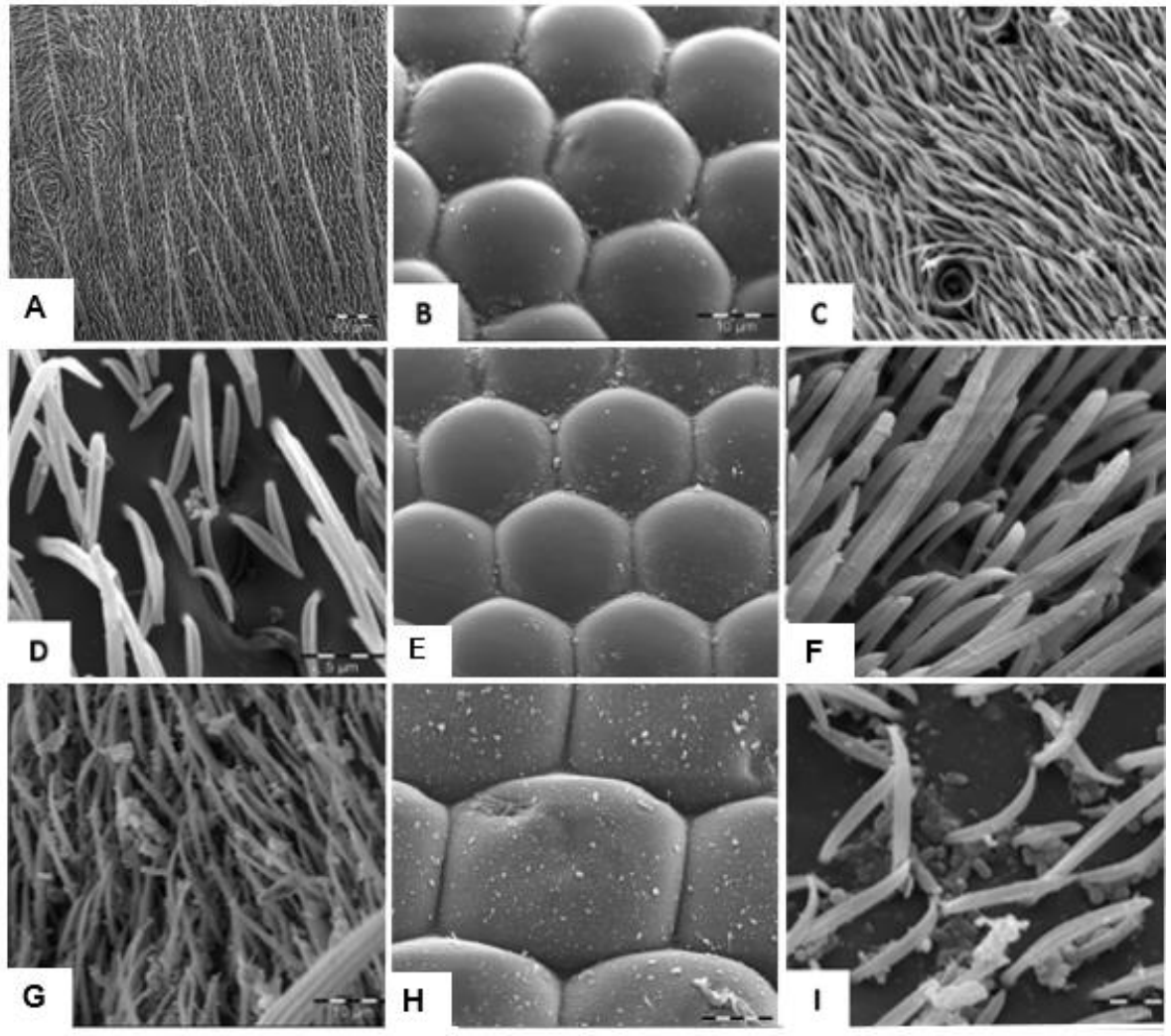


Plate 4.2: Scanning electron microscope (SEM) pictures. **A**: thorax (1 hr tween20), **B**: eyes (2 hrs tween20), **C**: abdomen (3 hrs tween20), **D**: thorax (1 hr bleach), **E**: eyes (2 hrs bleach), **F**: abdomen (3 hrs bleach), **G**: thorax (1 hr 70% EtOH), **H**: eyes (2 hrs 70% EtOH), **I**: abdomen (3 hrs 70%EtOH).

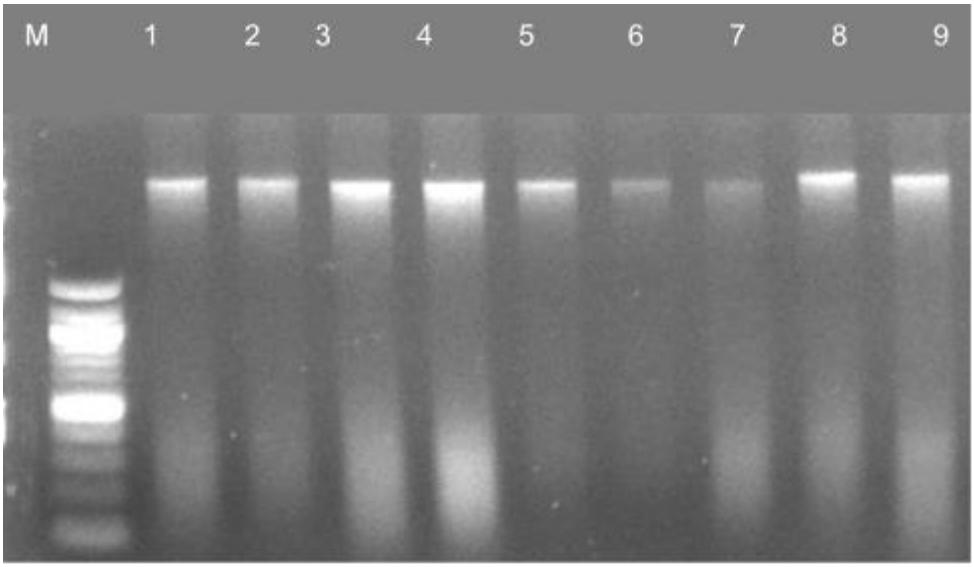


Figure 4.1: Gel electrophoresis image of extracted DNA from *S. calcitrans* after treatment with Tween 20, bleach and ethanol. **Lane M**: DNA ladder (100bp), **Lane 1**: 1 hr tween20, **Lane 2**: 2 hrs tween20, **Lane 3**: 3 hrs tween20, **Lane 4**: 1 hr bleach, **Lane 5**: 2 hrs bleach, Lane 6: 3 hrs bleach, **Lane 7**: 1 hour 70% EtOH, **Lane 8**: 2 hrs 70% EtOH, **Lane 9**: 3 hrs 70% EtOH

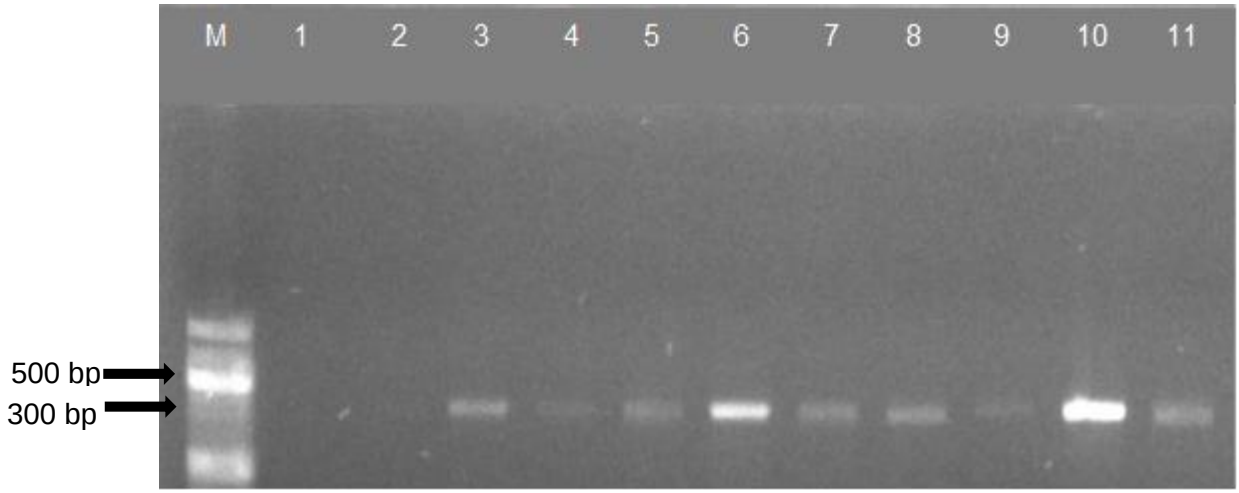


Figure 4.2: Gel image showing PCR amplification of a portion of the *16S rRNA* [300 bp] gene of *Stomoxys calcitrans.* **Lane M**: DNA ladder (100bp), **Lane 1**: negative control (ddH_20), **Lane 2**: negative control (mixture of 10% tween20, 10% bleach and 70% EtOH), **Lane 3**: 1 hr Tween20, Lane 4: 2 hrs Tween20, **Lane 5**: 3 hrs Tween20, **Lane 6**: 1 hr 10% bleach, **Lane 7**: 2 hrs 10% bleach, **Lane 8**: 3 hrs 10% bleach, **Lane 9**: 1 hr 70% EtOH, **Lane 10**: 2 hrs 70% EtOH, **Lane 11**: 3 hrs 70% EtOH

4.3 Amplification of the fly DNA

The *CO1* and *16S rRNA* genes were successfully amplified from stable fly DNA samples. The length of *CO1* and *16S* sequences were 710 and 300 bp (Figure 4.3 & 4.4), respectively and there was no variation in length of either set of primer sequences among specimens sampled from different provinces.

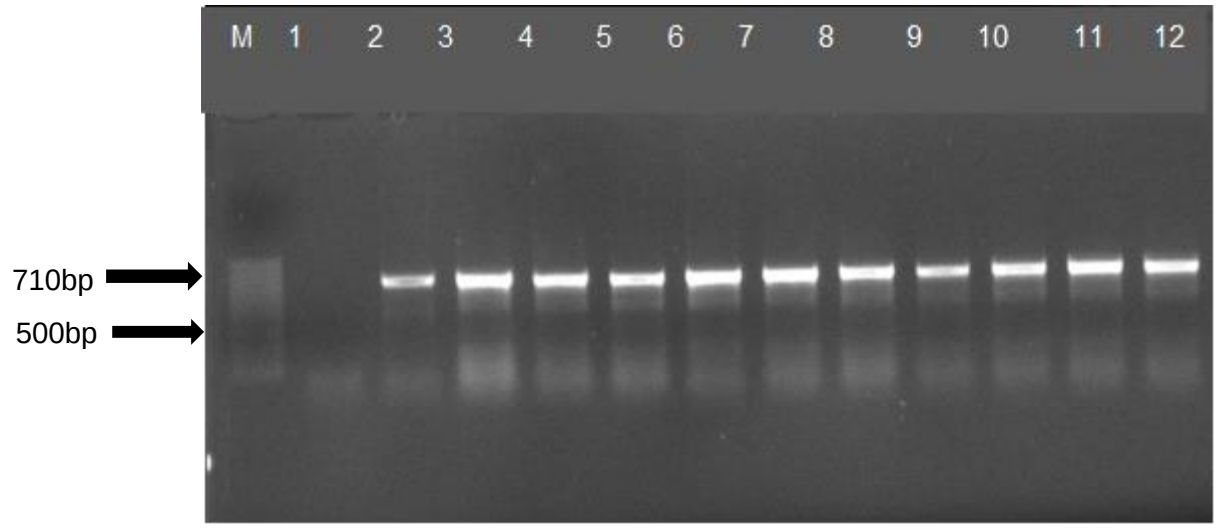


Figure 4.3: Gel image showing PCR amplification of a portion of mitochondrial *CO1* [710 bp] gene of *Stomoxys calcitrans.* **Lane M**: DNA ladder (100bp); **Lane 1**: negative control (ddH_20); **Lane 2**: positive control, **Lane 3-12**: *S. calcitrans* specimens. **Lane 3-6**:North West; **6-8** Free State and **9-12** Limpopo

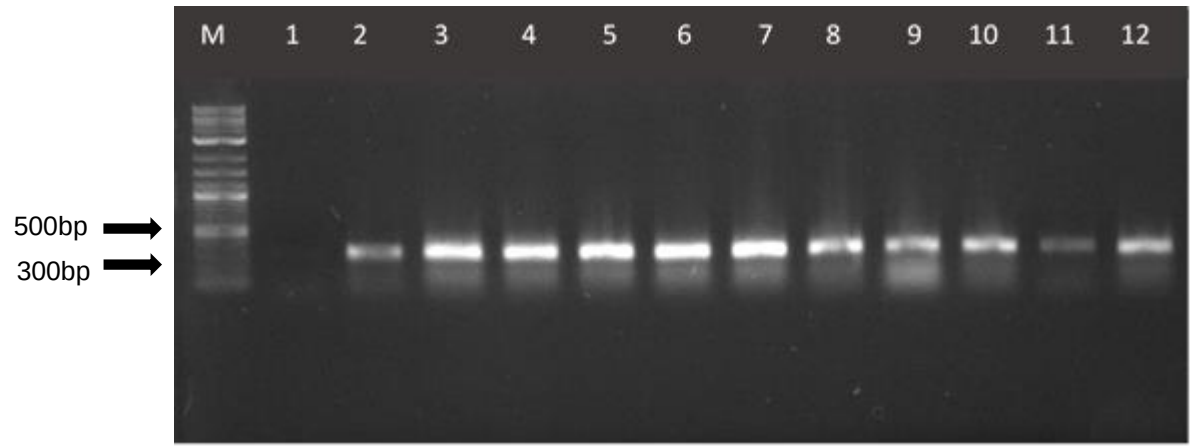


Figure 4.4: Gel image showing PCR amplification of a portion of the *16S rRNA* [300 bp] gene of *Stomoxys calcitrans.* **Lane M:** DNA ladder (100bp); **Lane 1:** negative control (ddH_20); **Lane 2:** positive control, **Lane 3-12:** *Stomoxys calcitrans* specimens. **Lane 3-6**:North West; **6-8** Free State and **9-12** Limpopo.

4.4 Phylogenetic analysis stable flies from South African feedlots

4.4.1 Analysis of *S. calcitrans CO1* gene

The average length of the sequenced *CO1* gene fragments for the South African stable flies was 687 bp. These sequences matched with the stable fly sequences available in GenBank when subjected to BLASTn (www.ncbi.nlm.nih.gov/blast). The sequences were 99% identical (Table 4.3) with published sequences of *S. calcitrans*.

One stable fly sequence was aligned against a reference sequence from GenBank and its nucleotide composition was analyzed. The analysis showed a total of 528 necleotide sequences with no gaps against the reference sequences. Figure 4.5 shows the nucleotide differences found in the *CO1* sequences of the stable fly species analysed. The average nucleotide frequencies excluding gaps were 29.3% adenine, 40.3% thymine, 15.3% cytosine and 15.5% guanine. The rates based on substitution for each nucleotide pair are shown in Table 4.4. Pairwise distance nucleotide differences found among the taxa included in this study are given in Table 4.5. From the table results, the sequence divergence among *Stomoxys* species is 0.084.

From the alignment of the two sequences of *S. calcitrans* shown in Figure 4.6, two polymorphic sites were observed with 1 gap and 1 transition. The sequences used for Maximum Likelihood (ML) phylogenetic analysis were from the genus of *Stomoxys* with *Amblyomma latepunctatum* used as an outgroup (Figure 4.7). Three major clades were resolved with high bootstrap support values. The North West sequences were not included in constructing the phylogenetic analysis due to the poor quality of the DNA.

Table 4.3. BLASTn results of *CO1* gene from stable flies

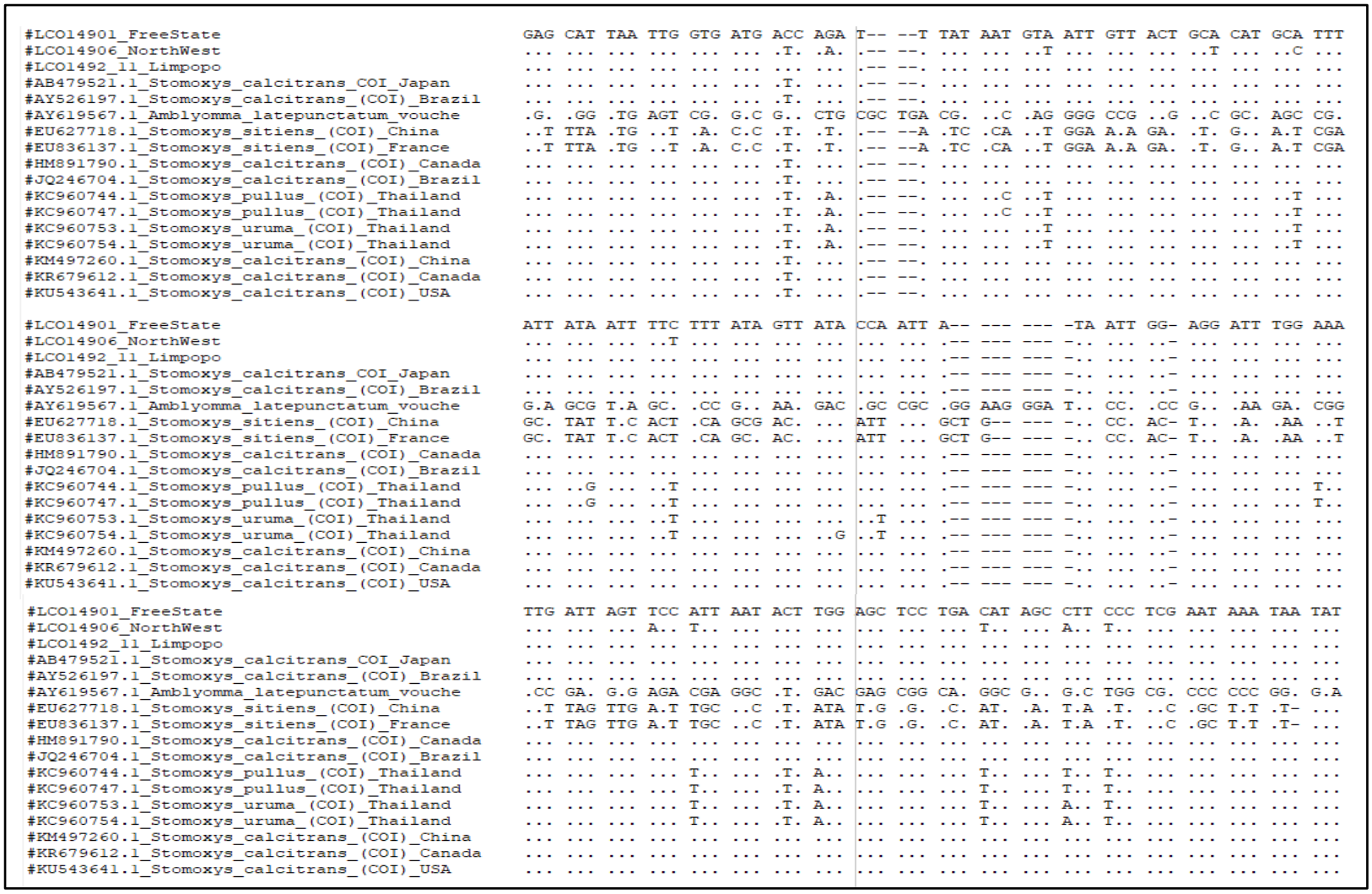
Gene	Morphological identification	BLASTn description	Maximum score	Total score	Query cover	E-value	Identity	Accession
CO1	*S. calcitrans*	*S. calcitrans*	1159	1159	100%	0	99%	KU543641.1
	S. calcitrans	*S. calcitrans*	1148	1148	99%	0	99%	KT272851.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	KU932147.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	JQ246704.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	EU627717.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	AB479521.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	AB479520.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	EU029769.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	DQ533708.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	0	99%	AY526197.1

Table 4.4. Stable flies *CO1* gene rates of base substitutions for each nucleotide pair

From/To	A	T	C	G
A	-	0.063694876	0.063694876	0.122610248
T	0.063694876	-	0.122610248	0.063694876
C	0.063694876	0.122610248	-	0.063694876
G	0.122610248	0.063694876	0.063694876	-

Table 4.5. : Stable flies *CO1* gene pairwise distance nucleotide differences found among taxa

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1. **LCO1_FS**		0.015	0.000	0.004	0.004	0.126	0.123	0.004	0.004	0.015	0.015	0.014	0.014	0.003	0.004	0.003
2. **LCO1_NW**	0.084		0.015	0.015	0.015	0.137	0.134	0.014	0.015	0.013	0.013	0.012	0.012	0.015	0.014	0.015
3. **LCO1_LP**	-0.000	0.084		0.004	0.004	0.126	0.123	0.004	0.004	0.015	0.015	0.014	0.014	0.003	0.004	0.003
4. *S. calcitrans*_AB479521.1	0.007	0.081	0.007		0.000	0.116	0.113	0.003	0.000	0.014	0.014	0.013	0.014	0.002	0.003	0.002
5. *S. calcitrans*_AY526196.1	0.007	0.081	0.007	-0.000		0.116	0.113	0.003	0.000	0.014	0.014	0.013	0.014	0.002	0.003	0.002
6. *S. sitiens*_EU627718.1	1.083	1.078	1.083	1.046	1.046		0.002	0.119	0.116	0.122	0.122	0.115	0.119	0.118	0.119	0.118
7. *S. sitiens*_EU836137.1	1.071	1.065	1.071	0.0134	1.034	0.002		0.117	0.113	0.119	0.199	0.113	0.117	0.116	0.117	0.116
8. *S. calcitrans*_HM891790.1	0.007	0.081	0.007	0.005	0.005	1.060	1.047		0.003	0.014	0.014	0.013	0.014	0.002	0.000	0.002
9. *S. calcitrans*_JQ246704.1	0.007	0.081	0.007	-0.000	-0.000	1.046	1.034	0.005		0.014	0.014	0.013	0.014	0.002	0.003	0.002
10.*S. pullus*_KC960744.1	0.084	0.070	0.084	0.076	0.076	1.062	1.049	0.076	0.076		0.000	0.010	0.010	0.014	0.014	0.014
11. *S. pullus*_KC960747.1	0.084	0.070	0.084	0.076	0.076	1.062	1.049	0.076	0.076	-0.000		0.010	0.010	0.014	0.014	0.014
12. *S. uruma*_KC960753.1	0.081	0.062	0.081	0.073	0.073	1.016	1.004	0.073	0.073	0.044	0.044		0.002	0.014	0.013	0.014
13.*S. uruma*_KC960754.1	0.084	0.065	0.084	0.076	0.076	1.028	1.016	0.076	0.076	0.047	0.047	0.002		0.014	0.014	0.014
14. *S. calcitrans*_KM497260.1	0.005	0.084	0.005	0.002	0.002	1.058	1.046	0.002	0.002	0.078	0.078	0.076	0.078		0.002	0.000
15. *S. calcitrans*_KR679612.1	0.007	0.081	0.007	0.005	0.005	0.060	1.047	-0.000	0.005	0.076	0.076	0.073	0.076	0.002		0.002
16. *S. calcitrans*_KU543641.1	0.005	0.084	0.005	0.002	0.002	1.058	1.046	0.002	0.005	0.078	0.078	0.076	0.078	-0.000	0.002	



```
#LCO14901_FreeState                    GAG CAT TAA TTG GTG ATG ACC AGA T-- --T TAT AAT GTA ATT GTT ACT GCA CAT GCA TTT
#LCO14906_NorthWest                    ... ... ... ... ... ... .T. .A. .-- --. ... ... ..T ... ... ... ..T ... ..C ...
#LCO1492_11_Limpopo                    ... ... ... ... ... ... ... ... .-- --. ... ... ... ... ... ... ... ... ... ...
#AB479521.1_Stomoxys_calcitrans_COI_Japan ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ... ...
#AY526197.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ... ...
#AY619567.1_Amblyomma_latepunctatum_vouche .G. .GG .TG AGT CG. G.C G.. CTG CGC TGA CG. ..C .AG GGG CCG ..G ..C GC. AGC CG.
#EU627718.1_Stomoxys_sitiens_(COI)_China ..T TTA .TG ..T .A. C.C .T. .T. .-- --A .TC .CA ..T GGA A.A GA. .T. G.. A.T CGA
#EU836137.1_Stomoxys_sitiens_(COI)_France ..T TTA .TG ..T .A. C.C .T. .T. .-- --A .TC .CA ..T GGA A.A GA. .T. G.. A.T CGA
#HM891790.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ...
#JQ246704.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ...
#KC960744.1_Stomoxys_pullus_(COI)_Thailand ... ... ... ... ... ... .T. .A. .-- --. ... ..C ..T ... ... ... ... ..T ...
#KC960747.1_Stomoxys_pullus_(COI)_Thailand ... ... ... ... ... ... .T. .A. .-- --. ... ..C ..T ... ... ... ... ..T ...
#KC960753.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ... ... ... .T. .A. .-- --. ... ... ..T ... ... ... ... ..T ...
#KC960754.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ... ... ... .T. .A. .-- --. ... ... ..T ... ... ... ... ..T ...
#KM497260.1_Stomoxys_calcitrans_(COI)_China ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ...
#KR679612.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ...
#KU543641.1_Stomoxys_calcitrans_(COI)_USA ... ... ... ... ... ... .T. ... .-- --. ... ... ... ... ... ... ... ... ...

#LCO14901_FreeState                    ATT ATA ATT TTC TTT ATA GTT ATA CCA ATT A-- --- --- -TA ATT GG- AGG ATT TGG AAA
#LCO14906_NorthWest                    ... ... ... ..T ... ... ... ... ... ... .-- --- --- -.. ... ... ... ... ... ...
#LCO1492_11_Limpopo                    ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#AB479521.1_Stomoxys_calcitrans_COI_Japan ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#AY526197.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#AY619567.1_Amblyomma_latepunctatum_vouche G.A GCG T.A GC. .CC G.. AA. GAC .GC CGC .GG AAG GGA T.. CC. .CC G.. .AA GA. CGG
#EU627718.1_Stomoxys_sitiens_(COI)_China GC. TAT T.C ACT .CA GCG AC. ... ATT ... GCT G-- --- -.. CC. AC- T.. .A. .AA ..T
#EU836137.1_Stomoxys_sitiens_(COI)_France GC. TAT T.C ACT .CA GC. AC. ... ATT ... GCT G-- --- -.. CC. AC- T.. .A. .AA ..T
#HM891790.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#JQ246704.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#KC960744.1_Stomoxys_pullus_(COI)_Thailand ... ..G ... ..T ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... T..
#KC960747.1_Stomoxys_pullus_(COI)_Thailand ... ..G ... ..T ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... T..
#KC960753.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ..T ... ... ... ... ..T ... .-- --- --- -.. ... ..- ... ... ... ...
#KC960754.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ..T ... ... ... ..G ..T ... .-- --- --- -.. ... ..- ... ... ... ...
#KM497260.1_Stomoxys_calcitrans_(COI)_China ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#KR679612.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...
#KU543641.1_Stomoxys_calcitrans_(COI)_USA ... ... ... ... ... ... ... ... ... ... .-- --- --- -.. ... ..- ... ... ... ...

#LCO14901_FreeState                    TTG ATT AGT TCC ATT AAT ACT TGG AGC TCC TGA CAT AGC CTT CCC TCG AAT AAA TAA TAT
#LCO14906_NorthWest                    ... ... ... A.. T.. ... ... ... ... ... ... T.. ... A.. T.. ... ... ... ... ...
#LCO1492_11_Limpopo                    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AB479521.1_Stomoxys_calcitrans_COI_Japan ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY526197.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY619567.1_Amblyomma_latepunctatum_vouche .CC GA. G.G AGA CGA GGC .T. GAC GAG CGG CA. GGC G.. G.C TGG CG. CCC CCC GG. G.A
#EU627718.1_Stomoxys_sitiens_(COI)_China ..T TAG TTG A.T TGC ..C .T. ATA T.G .G. .C. AT. .A. T.A .T. ..C .GC T.T .T- ...
#EU836137.1_Stomoxys_sitiens_(COI)_France ..T TAG TTG A.T TGC ..C .T. ATA T.G .G. .C. AT. .A. T.A .T. ..C .GC T.T .T- ...
#HM891790.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#JQ246704.1_Stomoxys_calcitrans_(COI)_Brazil ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#KC960744.1_Stomoxys_pullus_(COI)_Thailand ... ... ... ... T.. ... .T. A.. ... ... ... T.. ... T.. T.. ... ... ... ... ...
#KC960747.1_Stomoxys_pullus_(COI)_Thailand ... ... ... ... T.. ... .T. A.. ... ... ... T.. ... T.. T.. ... ... ... ... ...
#KC960753.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ... T.. ... .T. A.. ... ... ... T.. ... A.. T.. ... ... ... ... ...
#KC960754.1_Stomoxys_uruma_(COI)_Thailand ... ... ... ... T.. ... .T. A.. ... ... ... T.. ... A.. T.. ... ... ... ... ...
#KM497260.1_Stomoxys_calcitrans_(COI)_China ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#KR679612.1_Stomoxys_calcitrans_(COI)_Canada ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#KU543641.1_Stomoxys_calcitrans_(COI)_USA ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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Figure 4.5: Nucleotide differences found in the *CO1* gene sequences of stable fly species. A dot (.) indicates that the sequence at that point is identical to the reference sequence.

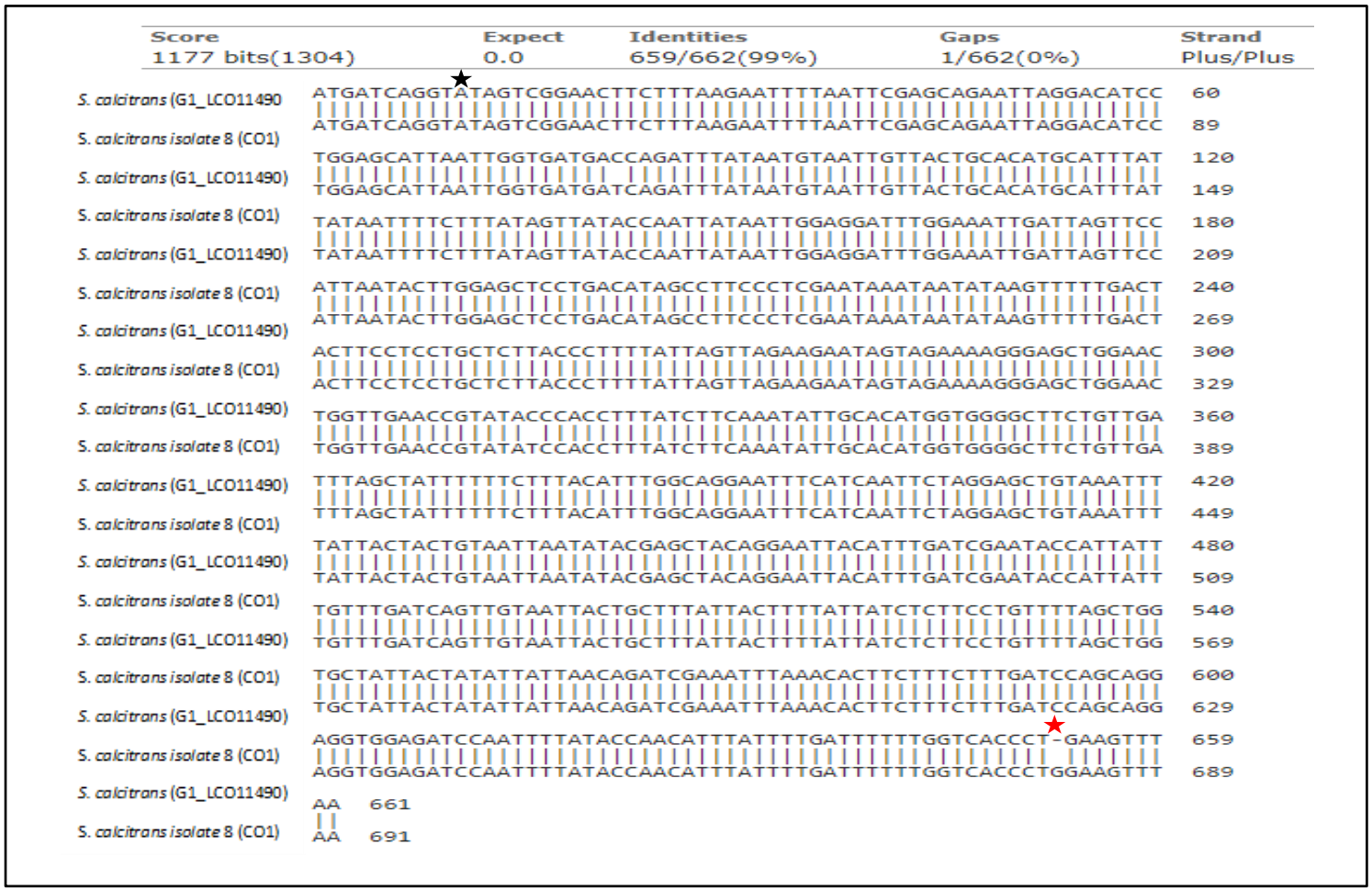

Figure 4.6: BLASTn results showing the alignment of *S. calcitrans* and one of the sequences from this study which was from a feedlot sample from Geysbert Hoek, Free State Province. The subject sequence matched with 99% of the query sequence (G1_LCO11490) and it had 99% match score with 1 gap and a maximum score of 1177. The black star indicates transversions as well as transitions that occurred between sequences and red star shows a gap between the two aligned sequences.

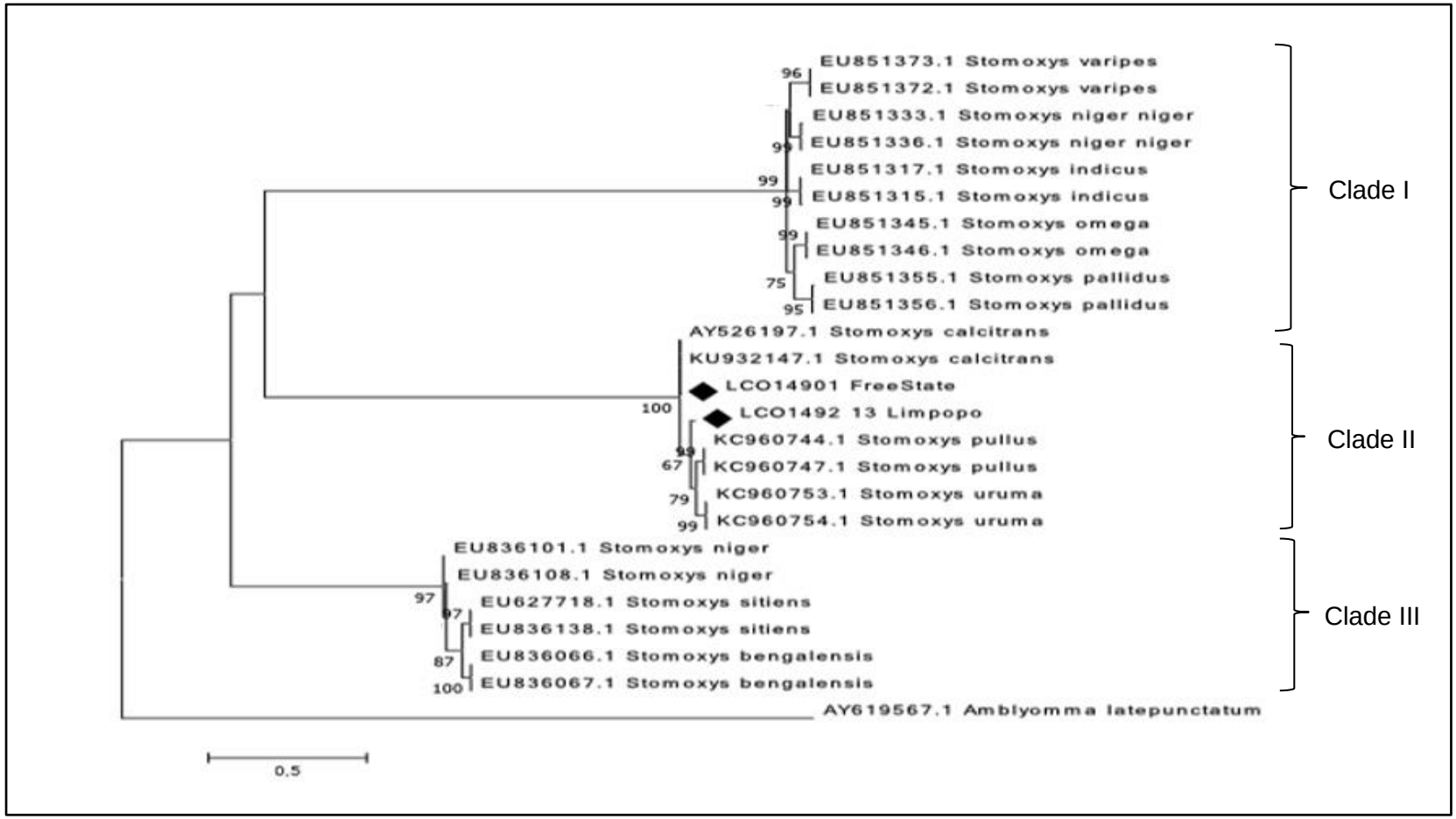

Figure 4.7: Molecular Phylogenetic analysis by Maximum Likelihood (ML) method of the *CO1* gene. The tree highlights the position of South African *Stomoxys* flies. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 536 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, 2016; Tamura *et al*., 2013)

4.4.2 Analysis of *S. calcitrans 16S rRNA* gene

The alignment of 16S rRNA gene sequences of the *S. calcitrans* species analyzed resulted in a total of 245 positions excluding gaps. The sequence of the stable flies from the current study matched with the sequences available in GenBank by BLASTn (http://www.ncbi.nlm.nih.gov/blast). The sequences generated from the current study were 99% to 100% identical (Table 4.6) with published sequences of *S. calcitrans*. The rates based on substitution for each nucleotide pair are shown in table 4.7. The *16S rRNA* gene was not subjected to any phylogenetic analysis due to the short length of the product size. From the alignment of the two sequences of *S. calcitrans* shown in figure 4.8, there were no polymorphic sites observed with no gaps, no transition and transversions. The average nucleotide frequencies excluding gaps were 40% adenine, 36% thymine, 12% cytosine and 9% guanine.

Table 4.6. BLASTn results of *16S rRNA* gene from stable flies

Gene	Morphological identification	BLASTn description	Maximum score	Total score	Query cover	E-value	Identity	Accession
16S rRNA	*S. calcitrans*	*S. calcitrans*	511	511	100%	4.00E-141	100%	KT272851.1
	S. calcitrans	*S. calcitrans*	511	511	100%	4.00E-141	100%	DQ533708.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245737.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245736.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245719.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245716.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245708.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245699.1
	S. calcitrans	*S. calcitrans*	499	499	97%	3.00E-137	100%	HM245696.1
	S. calcitrans	*S. calcitrans*	1142	1142	99%	3.00E-137	99%	HM245692.1

Table 4.7. Stable flies *16S rRNA* gene rates of base pair substitutions for each nucleotide

From/To	A	T	C	G
A	-	0.088239487	0.024826703	0.06134954
T	0.088239484	-	0.060134954	0.024826703
C	0.088239487	0.213732667	-	0.04867703
G	0.213732667	0.088239486	0.024826703	-

Figure 4.8: : BLASTn results showing the alignment of *S. calcitrans* and one of the sequences from this study which was from a feedlot sample from Geysbert Hoek, Free State Province. The subject sequence matched with 99% of the query sequence (G4_N1J1254F) and it had 99% match score with zero gaps and 511 maximum score. No observed transversions or transitions occurred between sequences.

4.5 Detection of pathogen DNA from *Stomoxys* flies

4.5.1 Amplification of *Anaplasma marginale* DNA

A total of 213 pooled fly DNA samples were screened for the presence of *A. marginale* DNA. The gel electrophoresis revealed amplification product size ranging between 1276-1290 bp as shown in figure 4.9. The occurrence of *A. marginale* was 16% (16/100) in North West (n=100) being the highest province with infection followed by Free State (n=100) with 10% (10/100). None of the samples (n=13) collected from Limpopo province tested positive for *A. marginale.*

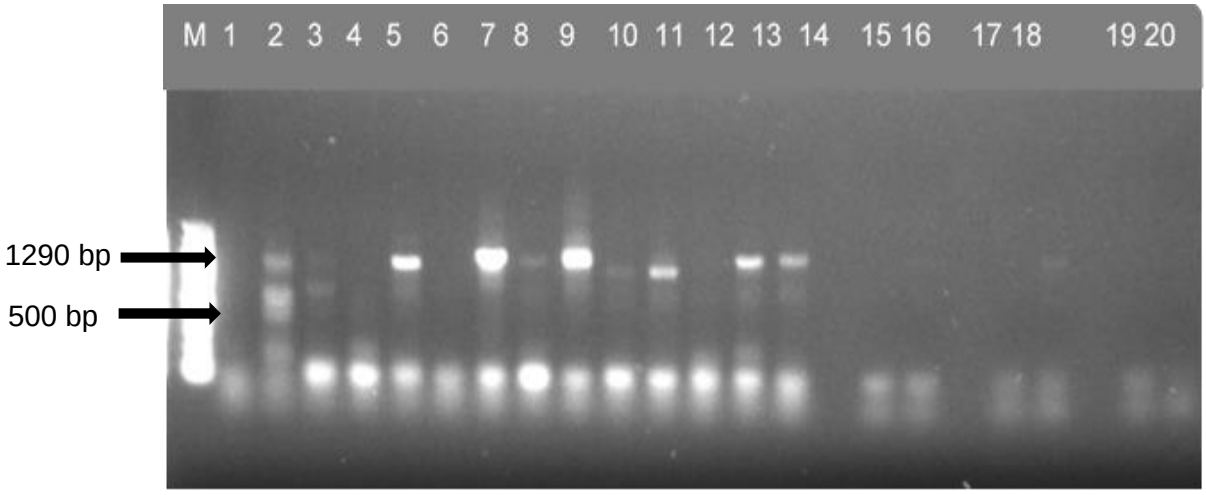

Figure 4.9: Gel image showing PCR amplification of *A. marginale* DNA from South African stable flies with amplicon size 1267-1290 bp. **Lane M:** DNA ladder (100bp); **Lane 1:** -ve control (dd$H_2$0); **Lane 2:** +ve control. **Lane 3-8** samples from Free State, **Lane 9-14:** samples from North West, **Lane 15-20** samples from Limpopo. **Lane 3, 5,7,8,9,10,11,13,14,** and **18** are PCR positive samples

4.5.2 Amplification of Lumpy skin disease virus DNA

A total of 213 pooled fly DNA samples were screened for the presence of LSDV DNA. The gel electrophoresis revealed amplification product size of 1237 bp as shown in figure 4.10. The occurrence of LSDV was 27% (27/100) in North West (n=100) being the only province with the infection detected. None of the samples collectes from Free State (n=100) and Limpopo (n=13) provinces tested positive for LSDV (table 4.8).

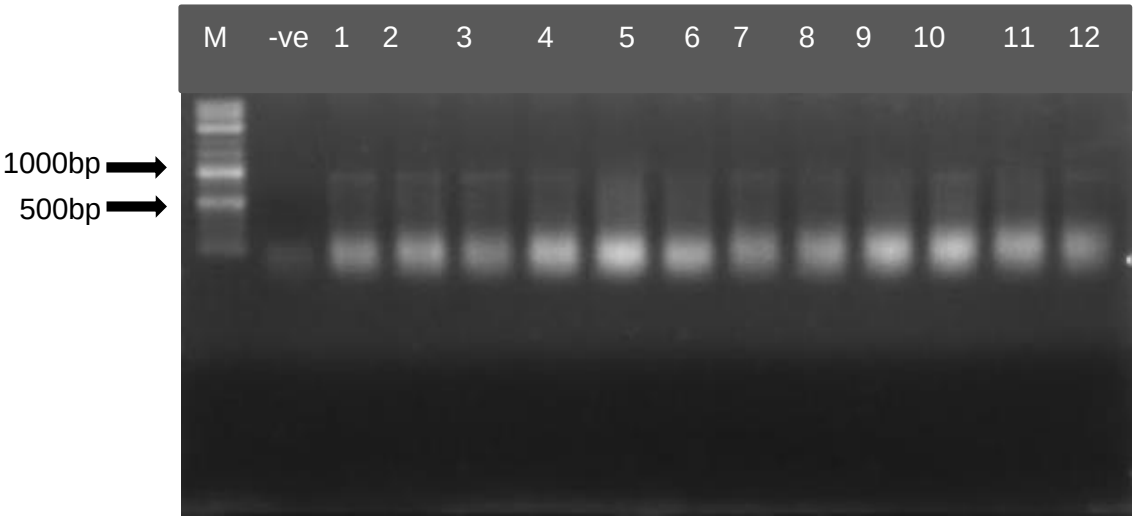

Figure 4.10: Gel image showing PCR amplification of Lumpy skin disease virus DNA from South African stable flies with amplicon size 1237 bp. **Lane M:** DNA ladder (100bp); **Lane 1:** -ve control (ddH_20); **Lane 1-12:** Samples from North West. **Lane 2, 3, 4, 6, 7, 8, 9, 10,** and **12** are PCR positive samples.

Table 4.8. Overall occurrence of pathogens in South African feedlots

Location	Total no. of samples	*A. marginale*	*Rickettsia*	*Ehrlichia*	*LSDV*
Potchefstroom	100	16%	0%	0%	27%
Sasolburg	100	10%	0%	0%	0%
Polokwane	13	0%	0%	0%	0%

4.6 Metagenomic analysis of South African stables flies

The South African stable flies gut microbe analysis was composed of 1 443 013 assembled sequences (Table 4.9). The sequences were trimmed into a total of 908 814. All the sequences were aligned against the reference SILVA bacteria for further analysis. A total of 462 OTUs per fly sample were produced in the final analysis.

Table 4.9. Number of sequences of South African stable fly samples used to produce OTUs

Sample_ID	Number of sequences aligned	Number of sequences assembled	Total number of OTUs
St_1V	77341	113858	462
St_2V	46672	76574	462
St_2G	31706	61988	462
St_3G	6385	20016	462
St_3V	175940	260315	462
St_5G	75098	151039	462
St_5V	261451	350069	462
St_8G	37575	92942	462
St_8V	145409	198619	462
St_9G	51237	117594	462
Total	**908814**	**1443014**	

4.6.1 Classification of microbiota detected from South African stable flies

From the three sampled provinces, only two were subjected to *16S rRNA* analysis. Samples collected from Polokwane (Limpopo Province) were not subjected to next generation sequencing due to the poor quality of the DNA.There was a total of six phyla recovered from Potchefstroom (North West) and Sasolburg (Free State), Sasolburg in South Africa. The most abundant phyla at Potchefstroom were Proteobacteria (48.3%), followed by Bacteroidetes at 10.7 %. Other bacterial phyla accounted for 24.0%, including all the bacterial phyla with less than 1% abundance At Sasolburg, the most abundant phyla were Proteobacteria (29.8%), followed by Actinobacteria at 10.0%. Other bacterial phyla accounted for 53.9%, and this included all bacterial phyla wih less than 1% abundance. The other important detected phyla (Figure 4.11) included Bacteriodetes, Firmicutes, Cyanobacteria, and Actinobacteria.

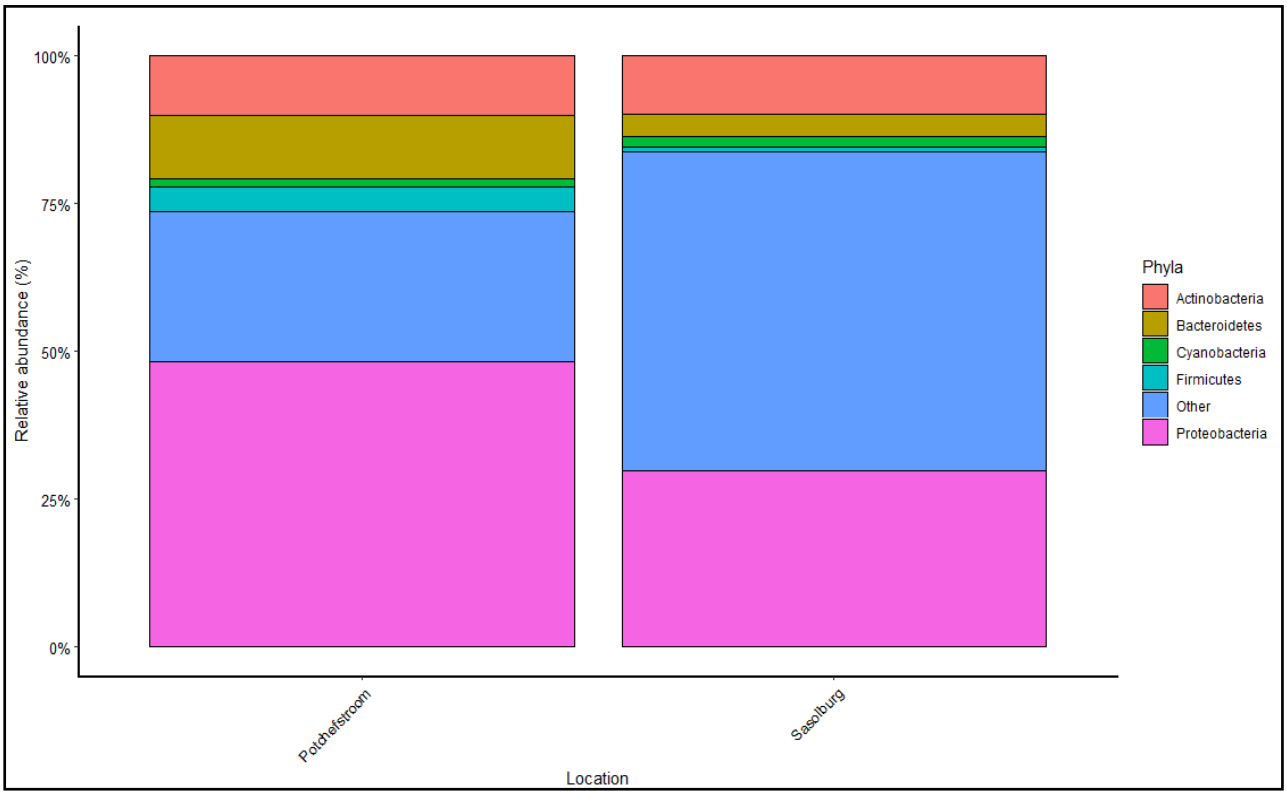

Figure 4.11: The proportion of bacterial phyla detected from Potchefstroom and Sasolburg stable flies

A total of 7 classes were recovered from Potchefstroom (North West Province) and Sasolburg (Free State Province). The most abundant classes at Potchefstroom were Alphaproteobacteria (47%), followed by Actinobacteria (9.8%). In Sasolburg, the most abundant classes were Alphaproteobacteria (29%), followed by Actinobacteria (9.8%). The sum total of all bacterial classes with less than 1% abundance were clustered together and grouped as a bacterial class (other) which amounted to 24.0% and 54.9% at Potchefstroom and Sasolburg respectively. The other important detected classes (Figure 4.12) included Clostridia, Bacteroidia, Saprospirae, and Chloroplast.

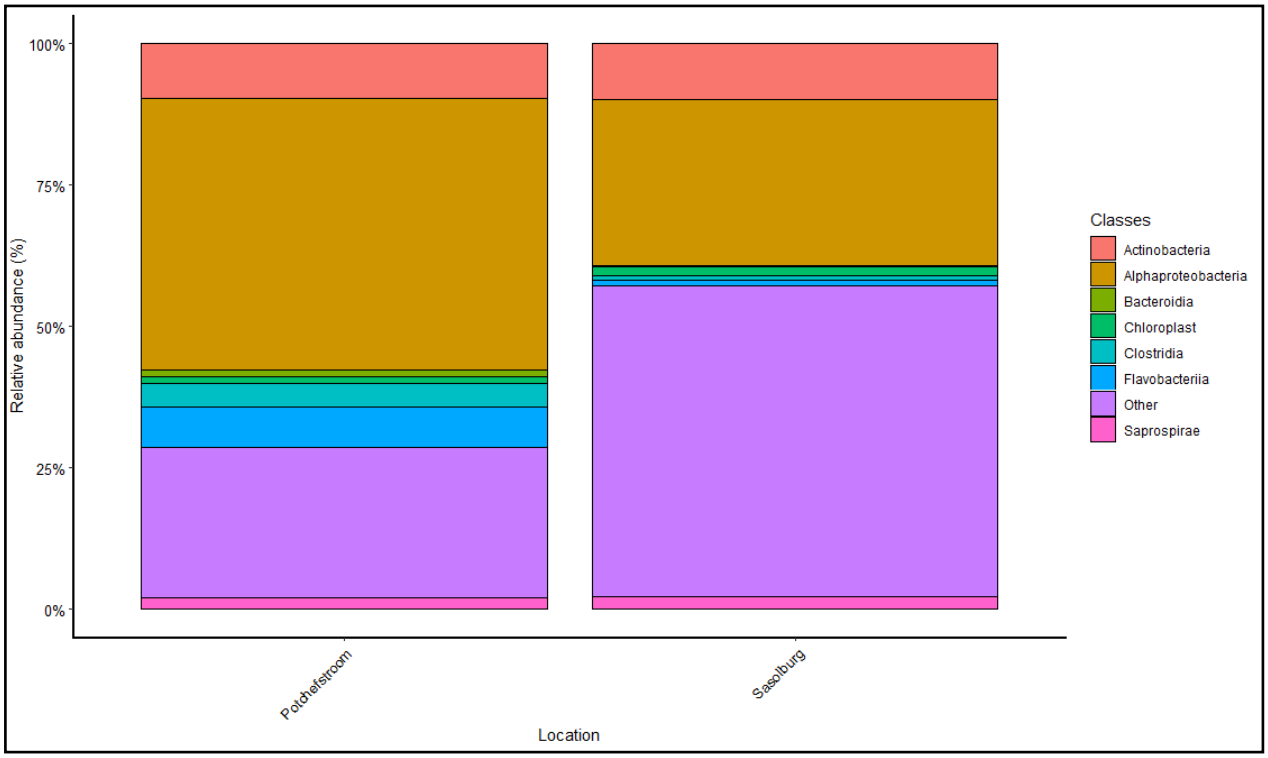

Figure 4.12: The proportion of bacterial classes detected from Potchefstroom and Sasolburg stable flies

A total of 11 orders were recovered from Potchefstroom (North West Province) and Sasolburg (Free State Province). The most abundant orders at Potchefstroom were Rickettsiales at 23.0%, followed by Sphigomonadales at 13.0% (Figure 4.13). In Sasolburg, the most abundant orders were Sphigomonadales (15.4 %), followed by Rhizobiales (11.7%). Sum total of all bacterial orders with less than 1% abundance were clustered together and grouped as a bacterial order (other) which amounted to 24.0 % and 51.9% at Potchefstroom and Sasolburg respectively. The other important detected orders that had the least abundance included Bacteroidales, Flavobacteriales, Actinomycetales, and Saprospirales.

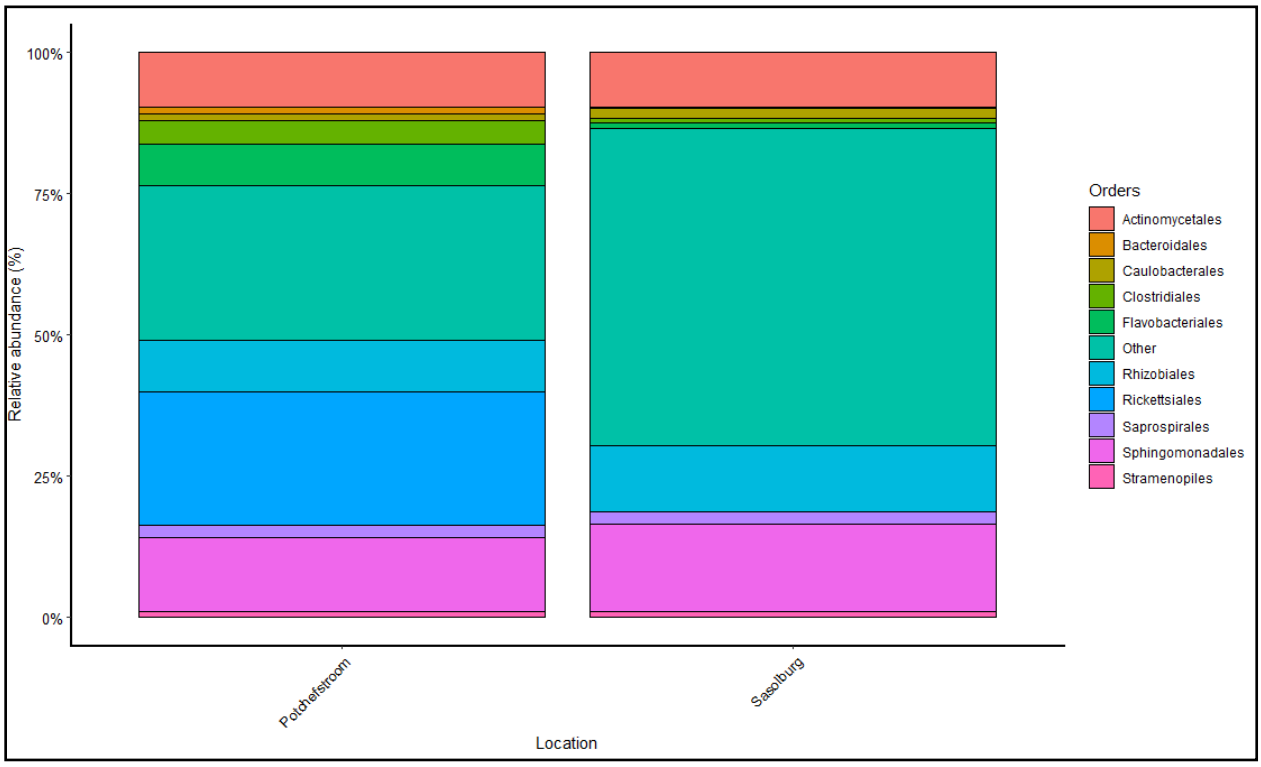

Figure 4.13: The proportion of bacterial orders detected from Potchefstroom and Sasolburg stable flies

A total of 15 families were recovered from Potchefstroom (North West Province) and Sasolburg (Free State Province). The most abundant families at Potchefstroom were Rickettsiaceae at 23%, followed by Sphingomonadaceae at 13.0% (Figure 4.14). In Sasolburg, the most abundant families were Sphingomonadaceae at 14.8 %, followed Bradyrhizobiaceae at 5.0%. Sum total of all bacterial families with less than 1% abundance were clustered together and grouped as a bacterial family (other) which amounted to 24.0 % and 51.9% at Potchefstroom and Sasolburg respectively. The other important detected families included Clostridiaceae, Flavobacteriaceae, Rhizobiaceae, and Methylobacteriaceae.

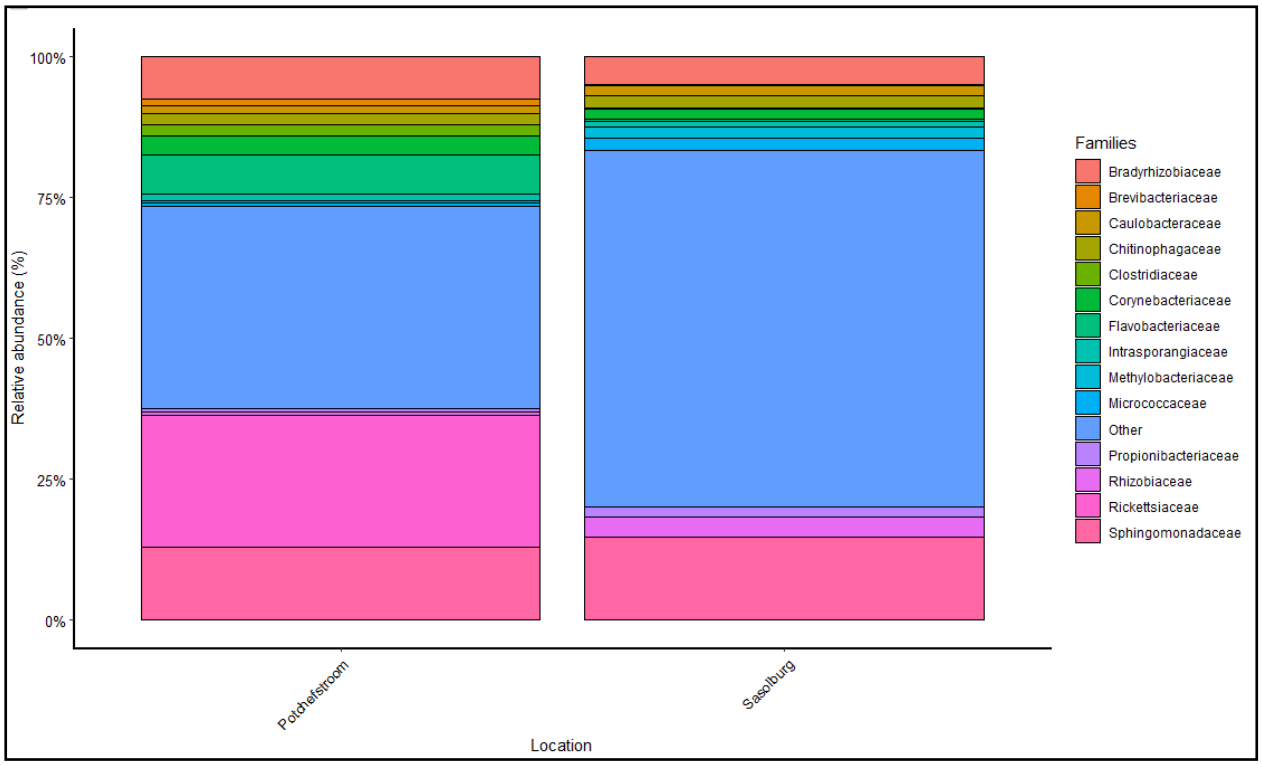

Figure 4.14: The proportion of bacterial families detected from Potchefstroom and Sasolburg stable flies

A total of 10 genera were recovered from Potchefstroom (North West Province) and Sasolburg (Free State Province). The most abundant genera at Potchefstroom were *Sphingomonas* at 12.1%, followed by *Wolbachia* at 11.7% (Fig 4.15). In Sasolburg, the most abundant genera were *Sphingomonas* at 13.4 %, followed by at *Agrobacterium* at 3.2%. The sum total of all bacterial genera with less than 1% abundance were clustered together and grouped as a bacterial genera (other) which amounted to 24.0 % and 51.0% at Potchefstroom and Sasolburg respectively. The other detected genera included *Flavobacterium*, *Methylobacterium*, *Brevibacterium*, and *Sediminibacterium*.

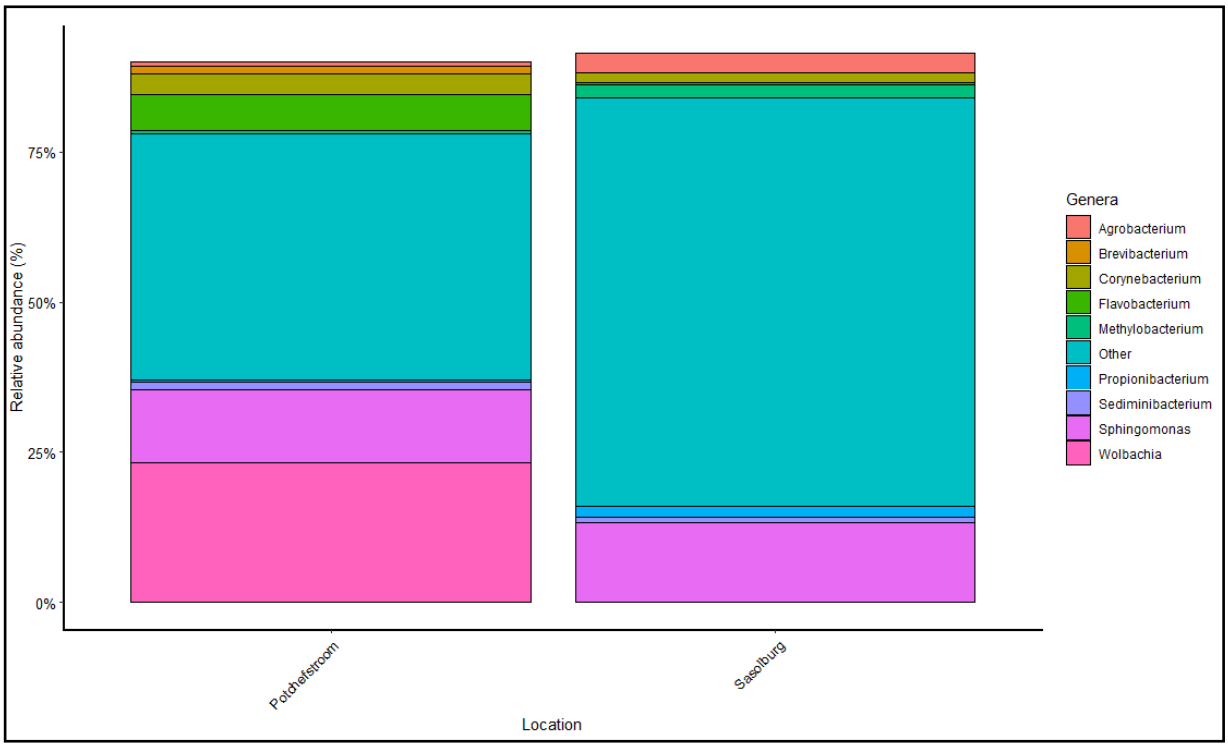

Figure 4.15: The proportion of bacterial genera detected from Potchefstroom and Sasolburg stable flies

4.6.2 *Alpha-beta* diversity of South African stable flies

The alpha diversity analysis was compiled to determine the species richness of the produced OTUs from calculated inverse Simpson diversity index for each fly sample is shown in Table 4.10. The Simpson's evenness index indicated that the bacterial community distribution in the samples were even, with similar bacterial diversity in the two locations. There was good coverage >99% observed in all the samples, indicating that the current sequencing depth was sufficient to saturate the bacterial diversity of each sampled location.

Table 4.10: The a*lpha*-diversity indices based on Illumina MiSeq data from South African stable flies

SampleID	Sampled_ Location	Chao1	Observed OTUs	Shannon diversity index	Simpson's evenness index	Good's Coverage
St_3V	Potchefstroom	2735	2547	6.33	0.949	99.70%
St_8V	Potchefstroom	2864	2713	6.09	0.922	99.67%
St_5V	Potchefstroom	2639	2442	3.64	0.627	99.80%
St_2V	Potchefstroom	1532	1197	5.89	0.933	99.17%
St_9G	Sasolburg	1049	889	4.78	0.889	99.49%
St_1V	Potchefstroom	1656	1384	5.09	0.876	99.46%
St_8V	Potchefstroom	1367	1292	6.95	0.968	99.41%
St_3G	Sasolburg	313	243	5.59	0.964	98.67%
St_2G	Sasolburg	702	596	5.84	0.962	99.45%
St_5G	Sasolburg	1100	1007	6.07	0.965	99.71%

Heatmaps and Venn Diagrams were compiled as additional analysis of the *beta* diversity. The *beta* diversity analysis was conducted to determine the relative abundance for the produced OTUs at class and genus level. The first heatmap is for relative abundance at class level and the second is for relative abundance at genera level between the two locations. Figure 4.16 clearly shows that there is a significantly high relative abundance of bacterial classes among the two sampled locations. Additionally, there was more than 90% sharing of bacterial communities between the two locations. In Figure 4.17 it is observed that *Wolbachia* was the most abundant genera in Potchefstroom, while *Agrobacterium* was the least. Similarly, in Sasolburg *Sphingonomonas* was the most abundant while *Brevibacterium* was the least bacterial genera. At both locations, *Sediminibacterium* and *Corynebacterium* showed equal number of bacterial genera. The sum total of all bacterial genera with less than 1% abundance were clustered together and grouped as a bacterial genera (other) was more abundant in Sasolburg compared to Potchefstroom. Other bacterial genera that had relatively low abundance in the two locations were *Propionobacterium*, *Methylbacterium*, *Brevibacterium*, and *Flavobacterium*.

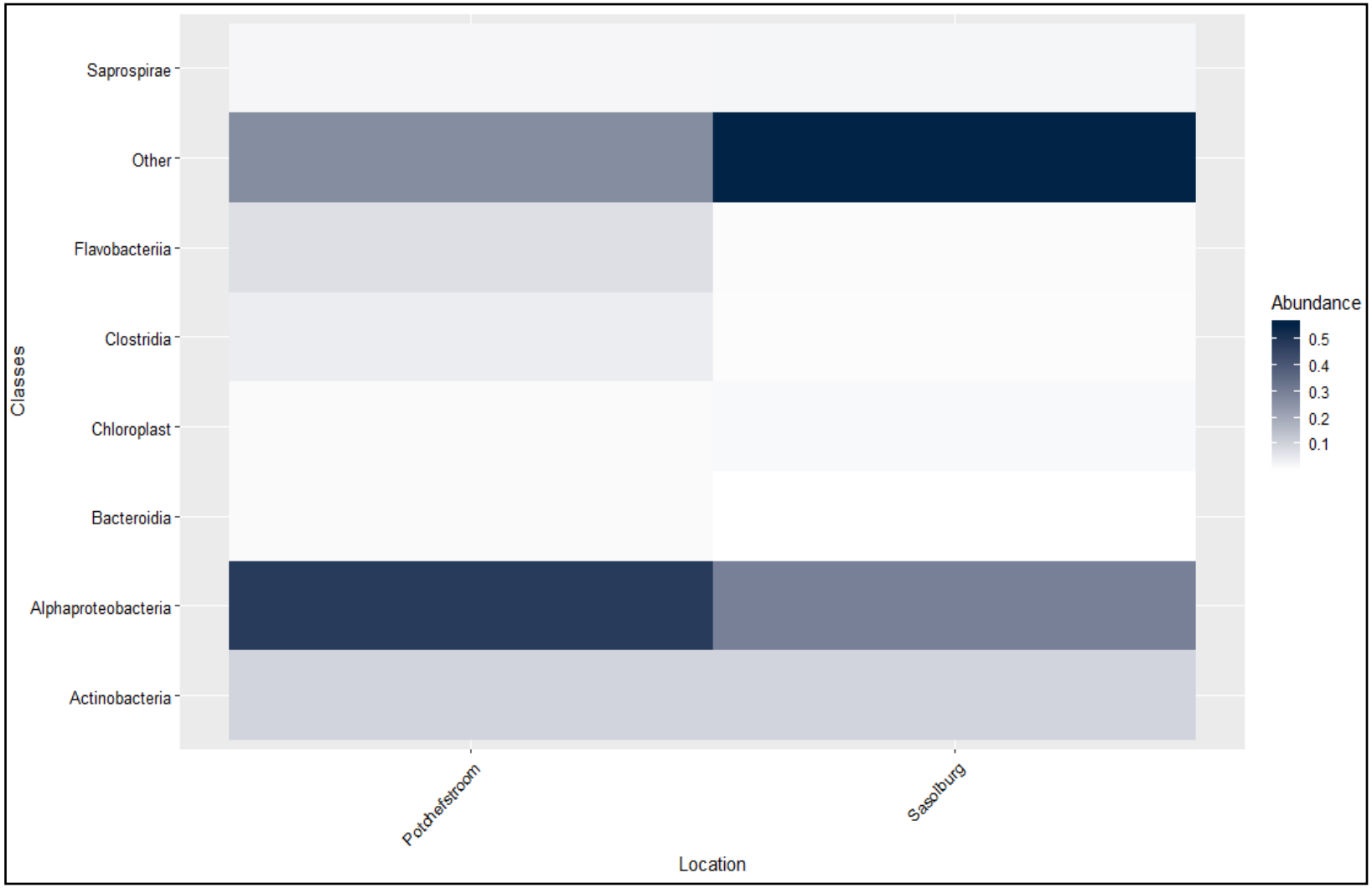

Figure 4.16: Heatmap at class level for South African stable flies

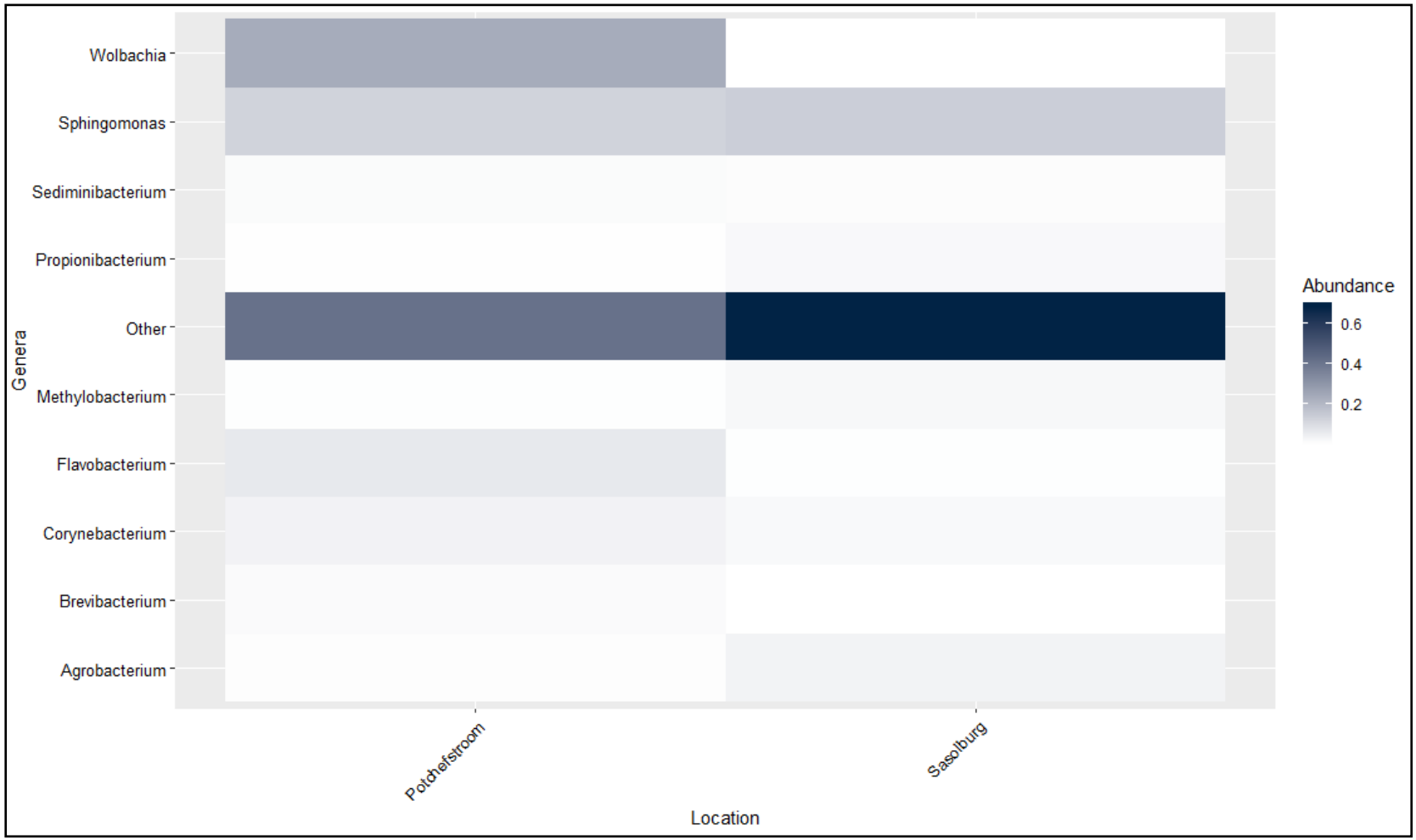

Figure 4.17: Heatmap at genus level for South African stable flies

The second part of the beta diversity based on the Venn diagram was used to determine shared number of species between the two sampled locations.

In Figure 4.18, Sasolburg contained 31 OTUs, and Potchefstroom contained 220 OTUs. A total of 209 bacterial species were shared between Sasolburg and Potchefstroom.

Figure 4.18: Venn diagram composed of two sampled locations

Bacterial genera of medical, veterinary and ecological importance detected in the current study include *Clostridium, Bacillus, Anaplasma, Rickettsia, Wolbachia, and Rhizobium* in Table 4.11.

Table 4.11. Bacterial genera of medical, veterinary and ecological importance as well as those in symbiotic associations with arthropod

Genera	Phylum	Family	Medical/Veterinary/Ecological/Symbiotic significance
Clostridium	Firmicutes	Clostridiaceae	Human and animal pathogens
Bacillus	Firmicutes	Bacillaceae	Human and animal pathogens
Enterococcus	Firmicutes	Enterococcaceae	Nosocomial infections in humans
Staphylococcus	Firmicutes	Staphylococcaceae	Nosocomial infection in humans
Streptococcus	Firmicutes	Streptococcaceae	Human and animal pathogens
Anaplasma	Proteobacteria	Erhlichiaceae	Animal pathogens
Rickettsia	Proteobacteria	Rickettsiaceae	Human and animal pathogens
Pseudomonas	Proteobacteria	Psuedomonadaceae	Human and plant pathogens
Wolbachia	Proteobacteria	Anaplasmataceae	Gut symbiont of insects
Neisseria	Proteobacteria	Neisseriaceae	Human pathogens
Mycoplasma	Tenericutes	Mycoplasmataceae	Human pathogens
Rhizobium	Proteabacteria	Rhizobiaceae	Symbiont of roots and legumes

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Characterization of South African *S. calcitrans*

More than 10 195 insect specimens from the orders Diptera, Hymoneptra, Lepidoptera, Isoptera, Coleoptera, Mantodea, Neuroptera as well as Orthoptera were captured in the current study using the Vavoua traps. The traps are durable and proved to be an effective method of collecting stable flies (Mihok *et al*., 1995; Gilles *et al*., 2005; Gilles *et al*., 2007), but can further be improved by adding acetone alone (Mihok *et al*., 1995) or in association with carbon dioxide (Mihok *et al*.,1996) to increase the trap efficiency. Catches results depend on the trapping methods and do not necessarily express the real abundance and distribution of flies in the field (Luff, 1975; Drach *et al*., 1981; Williams *et al*., 1990).

Stable flies belong to the genus *Stomoxys* with 18 described species (Foil & Hogsette, 1994; Muenworn *et al*., 2010; Keawrayup *et al*., 2012). The *S. calcitrans* was the only species recorded in the present study from the genus *Stomoxys*. Morphologically *S. calcitrans* appear to be similar in size with *Musca domestica* (5 mm – 7 mm) but *S. calcitrans* are easily recognized by their moderately bent fourth wing vein, grey thorax with longitudinal stripes, distinct checkered pattern on the abdomen and a thin long proboscis that protrudes from under the head as indicated in plate 4.1 (Howel *et al*., 1978; Foil & Hogsette, 1994; Masmeatathip *et al*., 2006; Tangtrakulwanich 2012). In the current study, other species of the genus *Stomoxys* were not encountered from all the sampled feedlots. Similarly, in a study conducted by Evert (2014) on temporal distribution and relative abundance of stable flies (*S. calcitrans*) in a feedlot near Heidelburg Gauteng, no other species of *Stomoxys* were recorded.

Sequenced PCR amplicons of the *CO1* and the *16S rRNA* genes of *S. calcitrans* matched with *S. calcitrans* sequences available on GenBank database with 99% -100% identity score which confirmed observations made with the morphological identification. Observations made from the maximum likelihood tree topology constructed with *CO1* gene showed three major clades which support monophyly within the tribe Stomoxyini.

These findings are similar to those by Dsouli and colleagues (2011). All the species studied clustered with corresponding congener. Sequences of *S. calcitrans* from this study formed a single group with those from GenBank as observed in clade II from Figure 4.7 Analysis generated from this study showed that the South African *Stomoxys* flies did form a well-supported clade with corresponding congener.

5.2 Surface sterilization of external microbes of *S. calcitrans*

Several antimicrobial agents have been used to sterilize fly surface for the removal of surface microbial communities. In the current study, hypochlorite (bleach), ethanol (EtOH), and polyoxyethylene (20) Sorbitan monolaurate (tween-20) were used to remove surface bacteria from the flies. Hypochlorite is the most widely used form of chlorine disinfectant and a large number of antimicrobially active chlorine compounds are available as sodium hypochlorite, calcium hypochlorite, liquid chlorine, and chlorine dioxide (Rutala & Weber,1997). They have a broad spectrum of antimicrobial activity and are inexpensive and fast. Alcohol refers to two water-soluble chemical compounds whose germicidal characteristics are generally underrated. These are ethyl-alcohol and iso-propyl alcohol. Alcohols are rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria; they are also tuberculocidal, fungicidal, and viricidal but do not destroy bacterial spores (Malik *et al*.,2006). Tween20 is polysorbate, non-ionic surfactant substance widely used as emulsifier (Dikici *et al*., 2013; Eskandani *et al*., 2013) and stabilizer in pharmaceutical formulations, detergents in domestic, and scientific applications (Dikici *et al*., 2013; Eskandani *et al*., 2013). Tween20 is also frequently added to washing buffers such as phosphate-buffered saline (PBS) and tris-buffered saline to prevent non-specific binding in radio-immuno assays and various techniques that employ the enzyme-linked immunosorbent assays (Zampieri *et al*.,2000; Eskandani *et al*.,2013).

Regarding all the treatment methods in this study, the scanning electron microscope (SEM) showed clear and distinct differences in terms of sterility as shown in plate 4.2. The differences between 10% bleach, 10% tween20, and 70% EtOH treatments of sterilized fly samples were observed under the SEM. However, there was no difference observed in soaking the samples for 1 hour, 2 hours, or 3 hours with each treatment. In the current study, 70% EtOH was one of the less effective disinfecting methods tested.

Microbial contaminants were seen adhering to the external surface of 70% EtOH treated fly samples. This was consistent with the findings of Greenstone *et al*., (2012) where 80% of EtOH reduced external contamination slightly in *Podisus* and not in *Coleomegilla* suggesting that washing with EtOH might not be effective in eliminating external DNA contamination in some circumstances. Studies by Brundage *et al*., (2016) demonstrated that only 57% of *Lucilia sericata* egg samples treated with 70% EtOH were disinfected. The same study further suggested that its poor performance was due to limited amount of time the EtOH was exposed to bacterial contaminants, while longer exposure might be more efficient but may also increase egg mortality. In this study, flies were subjected to up to 3 hours of 70% EtOH, and microbes were still seen adhering to the external surface of the flies.

The 10% bleach and 10% tween20 solutions appeared to be the most effective methods of sterilizing the external surface of stable fly without interfering with the analysis of the mtDNA of fly internal contents. Fly samples treated with 10% bleach and tween20 appeared to be smooth and clear of any external contaminants without destroying the host genomic DNA. Similarly, Meyer & Hoy (2008) concluded that bleach treatment reduced the quantity of external microbes while maintaining the endosymbiont DNA. Additionally, studies conducted by Linville & Wells (2002) demonstrated that surface sterilization of a maggot using bleach does not interfere with mitochondrial DNA analysis of crop contents. In this experiment, the *16S rRNA* primers were used to specifically amplify the host insects as confirmation. Each sterilization treatment did not interfere with PCR and amplification of host genomic DNA. Surface sterilization with different treatments can be used to reduce false positives for amplification of internal fly microbial DNA.

5.3 Pathogens harboured by South African stable flies

It is well recorded that stable flies are mechanical vectors of many pathogens such as those causing diseases LSD, trypanosomiasis, anaplasmosis, anthrax, besnoitiosis, West nile virus disease, Rift valley fever, bovine herpes virus disease, vesicular stomatitis, bovine leukosis, as well as equine infectious anaemia disease (Turell & Knudson, 1987; Scoles *et al*., 2005; Johnson *et al*., 2010; Lienard *et al*., 2012; Baldacchino *et al*., 2013). In the United States, it is estimated that the effects of stable

flies on livestock production amounts to US$ 1 million in economic losses annually (Kneeland *et al*., 2013; Taylor *et al*., 2012). This highlights the negative economic impacts that these flies pose on livestock production in heavily infested areas.

5.3.1 *Anaplasma*

Anaplasma marginale which causes bovine anaplasmosis is known to be mechanically transmitted by *Stomoxys* and tabanid flies. The biological vectors of *A. marginale* include ticks such as *Rhipicephalus decolaratus*, *R. microplus*, *Hyalomma marginatum rufipes*, *R. evertsi*, and *R. simu*s (Dreyer *et al*., 1998; de Waal, 2000). Bovine anaplasmosis is widespread throughout South Africa (Mutshembele *et al*., 2014), and has been reported to cause major economic losses in affected areas (Eriks *et al*., 1989; Rymaszewska & Grensa, 2008; Radwan *et al*., 2013). Cases of the disease have been reported in Gauteng, Mpumalanga, North West, Free State, Limpopo, Kwa-Zulu Natal, and Eastern Cape (Dreyer *et al*.,1998; Rikhotso *et al*., 2005; Ndou *et al*., 2010; Mutshembele *et al*., 2014). In the current study, the overall occurrence of *A. marginale* was 16% and 10% in stable flies from Potchefstroom and Sasolburg feedlots respectively. The detection of *A. marginale* from *S. calcitrans* from the sampled locations confirms the possibility of these flies acting as mechanical vectors for the pathogen. The geographical distribution of bovine anaplasmosis is not exclusively determined by its tick vector, in some regions of Latin America, the disease is more widespread than the *Rhipicephalus* (*Boophilus*) spp. This indicated the existence of other vectors or different modes of transmission, particularly mechanical transmission by biting flies and blood-contaminated fomites (Alonso *et al*., 1992). A study conducted by Scoles *et al*., (2005) clearly demonstrated that mechanical transmission of *A. marginale* by stable flies is highly possible. The study provided evidence that the Florida strain of *A. marginale* which is not transmittable by ticks, was more efficiently retained in stable fly mouth parts than the St. Maries strain, which is tick transmittable (Baldacchino *et al*.,2013). A survey by Olivera *et al*., (2011) indicated the correlation between the seroprevalence of *A. marginale* in dairy herd of Costa Rica and the presence of stable flies. Similarly, in recent studies and related studies, *S. calcitrans* has been associated with an outbreak of bovine anaplasmosis in Brazil (Machado *et al*., 2015). Studies by Bautista *et al*., (2018) have clearly demonstrated the mechanical transmission of *A. marginale* by *S. calcitrans* on tick free bovine herd, suggesting an active role in the

mechanical transmission of *A. marginale.* Results in the present study suggest that *S. calcitrans* are harbouring *A. marginale* and are possibly transmitting the disease in bovines from the sampled feedlots.

5.3.2 Lumpy Skin Disease Virus

LSDV was first reported in Zambia in 1929, and now is endemic in most of Sub-Saharan African parts of North Africa and has been reported in the middle east (Baldacchino *et al*., 2013; Saegerman *et al*., 2018). LSDV is purely mechanically transmitted by vectors such as stable flies (*S. calcitrans*), horse flies (tabanidae), mosquitoes, and ticks (Carn & Kitching, 1995; Chihota *et al*., 2001, 2003; Saegerman *et al*., 2018). This is supported with previous observations that associated most outbreaks with high abundance of biting flies (Von Backstrom 1945; Weiss, 1968). This study reports for the first time ever, the detection of LSDV from stable flies in South Africa. Ticks such as *Amblyomma hebraeum*, *Rhiphicephalus appendiculatus,* and *R. decolaratus* are well documented in the transmission of LSDV (Tuppurainen *et al*., 2013; Lubinga *et al*., 2013, 2014b). Stable flies have been shown to transmit sheep and goat poxvirus from infected to susceptible sheep and goats; and are therefore, the stable fly is considered to be a major vector of LSDV (Mellor *et al*., 1987). In the study conducted by Chihota *et al*., (2003) on the attempted mechanical transmission of LSDV by biting insects, stable flies (*S. calcitrans*) failed to transmit the virus to susceptible animals. However, this study only attempted transmission 24 h later than transmission period described by Webb (1990). It is therefore possible that *S. calcitrans* acts as mechanical vector of LSDV through interrupted feeding over 1 – 12 h, though not over longer periods. Furthermore, *S. calcitrans* has been implicated in the first LSDV outbreak in Israel in 1993 (Yeruham *et al*., 1995), suggesting that *S. calcitrans* were responsible for the transmission of the LSDV. Additionally, a study conducted by Kahana-Sutin *et al*., 2017 demonstrated that there was a significant correlation between LSDV outbreak and *S. calcitrans* abundance, where the *S. calcitrans* population peaked in the months of the lumpy skin disease onset. The current study presents the detection of LSDV from *S. calcitrans* collected from sampled feedlots (Van der Leeuw Boerdery, Doornbult and GysbertHoek feedlots in Potchefstroom, Limpopo and Sasolburg respectively) and supports the role of *S. calcitrans* as a potential mechanical vector of

LSDV in the sampled feedlots and this may ultimately lead to a decrease in the meat production sector in the country.

5.3.3 *Rickettsia* and *Ehrlichia*

Rickettsia and *Erhlichia* are tick-borne pathogens that infect animals and humans. There is ample literature available on the transmission of *Erhlichia* and *Rickettsia* spp. by ticks. However, there is a lack of information on the transmission of the pathogens by *S. calcitrans.* Stable flies (*S. calcitrans*) are recognized as mechanical vectors of a number of pathogens that can cause diseases in animals (Baldacchino *et al*.,2013). The current study attempted to detect the presence of *Ehrlichia* spp. from stable flies collected from feedlots in South Africa using PCR. Despite repeated attempts to detect the pathogen, all samples from the sampled locations were PCR negative. It was demonstrated by Burg *et al*., (1994) that *S. calcitrans* are able to ingest and retain viable *Ehrlichia risticii* although they are unable to transmit the pathogen. Studies by Levine *et al*., (1992) on field collected tabanids showed that they were capable of transferring *E. risticii* from infected to non-infected media by contaminated mouth part; however, failed to transfer the pathogen from an infected to a non-infected host. According to Ristic & Huxsoll (1984), the ehrlichae are obligate intracellular parasites, and successful mechanical transmission requires the transfer of intact infected cells between hosts. This suggested that, even though *Ehrlichia* may remain viable on mouthparts of some biting flies, it does not guarantee that the flies are capable of parasite transmission. Additionally, Levine *et al*., (1992) also suggested that although numerous biting flies have been well documented in the transmission of pathogens, no *Ehrlichia* spp. is known or documented to be transmitted by flies. In the current study no *Ehrlichia* spp. were detectable from stable flies to even suggest possible transmission. It is therefore possible that flies were not exposed to *Ehrlichia* hence there was no detection of the parasite or cattle in the sampled feedlots since they were free from *Ehrlichia* spp. infections.

Rickettsia spp. are regarded as emerging tick-borne pathogens, but so far data on the prevalence rates in biting flies *S. calcitrans* are rarely available. Although *Rickettsia* spp. were not detectable by PCR in the current study, it was detectable by metagenomics analysis indicating the titter of the rickettsial DNA was below the sensitivity of the high-

fidelity PCR protocol used in this study and thereby demonstrating the superior detection sensitivity of shotgun metagenomic analysis. Mechanical transmission of *Rickettsia* spp. is reported to occur when a fly feeding on a host with a patent infection is interrupted and the arthropods move to another susceptible host (Levine *et al*., 1992). The capacity of a vector to transmit certain pathogens is determined by its competence and by the probability of its biting and infecting a host. The probability of biting is related to host preference of the vector to vector abundance (Kahana-Sutin *et al*., 2017). The potential ability of *S. calcitrans* to mechanically transmit pathogens also depends on the level of parasitemia in the host blood stream and the time in between interrupted feeding. In *S. calcitrans,* the interval between blood meals varies from 4 to 72h. Thus, the regurgitation of the infected blood from the insect's crop can easily establish transmission and infection of pathogen (Baldacchino *et al*., 2013).

5.4 Microbiota of stable flies (Diptera: Muscidae) from South African feedlots

In this study, we present a survey of bacterial communities in *S. calcitrans* from South Africa obtained using next generation sequencing (NGS) technology. These flies were sampled from two feedlots in South Africa, namely, Van der Leeuw Boerdery and GysbertHoek feedlots in Potchefstroom and Sasolburg respectively. Stable flies satisfy their nutritional requirements as ectoparasites, requiring blood for development and reproduction (Schofield & Torr, 2002). Pathogens are transmitted to the host when flies suck blood and cause disease to the host (Butler *et al*., 1977; Doyle *et al*., 2011). However, despite the threat they pose, microbes associated with stable flies are poorly studied particularly in the African continent. It is therefore critical to study microbial community structure in *S. calcitrans* to control fly-borne diseases.

One of the objectives of the current study was to detect bacterial communities associated with stable flies by *16S* metabarcoding using NGS. From a total of 1 443 013 sequences retrieved from two sampled locations (the Free State and North West feedlot), there was a total of 462 OTUs documented which are composed of 6 phyla, 7 bacterial classes and 11 bacterial orders recovered with a total of 209 species richness between the sampled locations. The dominant phyla detected from data analysis of sampled locations included Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, and Cyanobacteria. These microbial phyla were consistent with data from many

previous studies on gut microbial communities associated with insects (Kikuchi, 2009; Jones *et al*., 2013; Gupta *et al*., 2014; Sabree & Moran, 2014). Some of these symbiotic bacteria are harmful posing as pathogenic parasites, while others are in mutualistic relationship with the host, providing nutrients and protection (Kikuchi, 2009; Jones et al., 2013; Sabree & Moran, 2014). Furthermore, a study by Scully *et al*., (2017) also indicated that these bacterial phyla were present in the larval stage and development substrate of stable flies which were also associated with other vertebrate animals including humans. This suggest that these phyla have ecological features that allow them to frequently reside within the animal host and vectors making them "core microbiomes" associated with animals (Jones *et al*., 2013).

Proteobacteria is the most common primary insect symbiont, which explains the high abundance of this phyla within insect relative to mammals (Jones *et al*., 2013). Hence, its high abundance in the analyzed stable flies. The bacterial diversity of stable fly was also comparable with that of other dipteran flies (Gupta *et al*., 2014), showing abundance of bacterial phyla Actinobacteria and Bacteriodetes and revealed relatively high abundance of genera such as *Corynebacterium*, *Propionibacterium*, and *Bacillus*. This corresponds with our findings in adult stable flies (*Stomoxys calcitrans*) and indicating that bacterial species of these genera are commonly associated with various fly species.

The genus *Wolbachia* was one of the genera detected in high abundance in Potchefstroom and Sasolburg stable flies in this study. Similarly, a study by Palavesam *et al*., (2012) on the analysis of the microbiome associated with horn fly *Haematobia irritans* revealed high abundance of *Wolbachia*, which are intracellular bacteria naturally present in large numbers of insects and other arthropod species (Palavesam *et al*., 2012). Insect vectorial capacity is influenced by *Wolbachia* infection, as it is known to inhibit virus replication inside insect vectors (Frentiu *et al*., 2010). *Wolbachia* also reduces the life span of insects which ultimately impacts their vectorial capacity (McMeniman *et al*., 2009). Furthermore, *Wolbachia* also ahs the capacity to increase the antioxidant and reactive oxygen's species level in insects (Brennan *et al*.,2008). *Rickettsia* spp. was one genus that was also detected from the sampled locations by *16S rRNA* amplicon pyrosequencing. However, no samples were positive with *gltA* gene PCR. This may be due to the fact that the titter level of the rickettsial DNA was below

the sensitivity of the high-fidelity PCR protocol used in this study. Thereby, demonstrating the high sensitivity of next generation sequencing platform (Qiu, 2014) as compared to conventional PCR.

The genera *Clostridium*, *Bacillus*, *Enteroccocus*, *Staphyloccocus*, *Streptococcus* identified in this study have already been described in some arthropods such as mosquitoes, ticks, and other fly species (Gusmao *et al*., 2010; Palavesam *et al*., 2012; Singh *et al*., 2015; Qui *et al*., 2014). The genus *Bacillus* is the largest and best-known member of the family Bacillaceae (Priest *et al*., 1988). This genus is a gram-positive, rod shape, endospore- forming bacteria of the phylum Fermicutes (Shida *et al*., 1997; Fritze, 2004). Various insects have been reported to carry different species of *Bacillus*. The *Bacillus anthracis* causes serious zoonotic diseases (Baldachhino *et al*., 2013). Herbivores become infected when they ingest sufficient numbers of spores in soil or on plant pesticides. However, stable flies and other dipterans have been implicated in the mechanical transmission of *B. anthracis* (Baldacchino *et al*., 2013). Turell & Knudson (1987) demonstrated that stable flies (*S. calcitrans*) can mechanically transmit *B. anthracis.* Furthermore, Schuberg & Kuhn (1912) were able to infect mice and guinea pigs with *B. anthracis* using *S. calcitrans* as a vector. These flies are well documented in the transmission of *B. anthracis*, which is considered to cause serious zoonotic diseases (Baldacchino *et al*., 2013). However, there is no documented information on the role played by this bacterium in flies.

Members of the genus *Enterobacter* have also been associated with *S. calcitrans.* The *E. sakazakii*, which is an opportunistic food-borne pathogen causing meningitis, enterocolitis, and sepsis (Hamilton *et al*., 2003; Baldacchino *et al*., 2013) was reported in adult stable flies. This poses a possibility of the flies contaminating their food source with this pathogen. *Stomoxys calcitrans* are considered as biological vectors of *E. sakazaki* as this bacterium has been found colonizing the gut of stable flies and surviving during stable fly development (Baldacchino *et al*., 2013). The *S. calcitrans* are cosmopolitan and the geographic distribution of stable flies correlates well with the incidence of *E. sakazakii* (Hamilton *et al*., 2003). Both *S. calcitrans* and *E. sakazaki* have been reported in Denmark, United States, Israeli, United Kingdom, and Germany (Hamilton et al.,2003). We observed *Staphylococcus* in our samples, which was also detected in previous study by Castro *et al*., (2007) where they reported the existence of

33 distinct species of bacteria in stable flies. These bacterial species were isolated from the cuticle, mouthparts, and abdominal alimentary tract of stable flies and included bacterial species members of the genus *Staphylococcus* such as *S. aureus* and *S. intermemedius*. *Staphylococcus aureus* are capable of causing various infectious diseases in man and animals by inducing toxigenic food poisoning and some other infectious diseases (Ako-Nai et al., 1991). These findings show that such bacteria are widespread in insects, suggesting that they can maintain a stable association with these insects. Our study also reports on the detection of various bacteria including *Clostridium*, *Streptococcus*, *Neisseria,* and *Mycoplasm*a which also constitutes environmental pathogenic bacteria.

5.5 Conclusion

This study has shown that stable flies are possibly harbouring *A. marginale* and LSDV. However, further studies should be carried out to determine the vectorial capacity of these flies for the transmission of *Rickettsia* spp. and *Ehrllichia* spp. The presence of *A. marginale* and LSDV DNA from South African stable flies needs further confirmation and the virulence of the two pathogens needs to be investigated.

This is the first study to characterize the internal microbiota harboured by stable flies in South Africa. Results from this study demonstrated the diversity of microbial communities from stable flies. Bacterial genera of medical, veterinary, and ecological importance were also recorded from this study. The relationship between these microorganisms and their arthropod host are not clear, and their potential to act as causative agents of emerging fly-borne mammalian disease warrants further investigation.

This study also provides a simple post collection and pre-gut content analysis sterilization protocol which offers an effective solution to the risk of external DNA contamination. All the treatments did not reduce the integrity of the insect's genomic DNA. However, EtOH was not considered a successful method of sterilization. It is therefore suggested that flies should be washed or sterilized with either 10% bleach or 10% tween20 followed by 70% EtOH and finally wash with distilled water.

5.6 Recommendations

- Future studies should be conducted on the characterization of *Stomoxys* spp. in other provinces of South Africa to determine the relative abundance of these flies in different geographic locations.

- There is a need to investigate the occurrence of detected pathogens from blood collected from cattle in sampled feedlots in order to elucidate the prevalence in mammalian hosts as compared to the stable flies as possible vectors.

- Species-specific PCR must be conducted to elucidate specie variation of medical and veterinary importance which are commonly revealed by metagenomics analysis.

- This study recommends that arthropod specimens be sterilized/washed with either 10% tween20 or 10% bleach in order to ensure removal of external microbial flora before detection of internal flora.

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APPENDIX I: OTU PRODUCED FROM SOUTH AFRICAN STABLE FLIES

OTU	PHYLUM	CLASS	ORDER	FAMILY	GENUS
1	Euryarchaeota	Methanobacter+C2:C400ia	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
2	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanosphaera
3	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
4	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
5	Euryarchaeota	Methanomicrobia	YC-E6		
6	Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae	
7	Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae	Edaphobacter
8	Acidobacteria	Solibacteres	Solibacterales		
9	Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075	
10	Actinobacteria	Acidimicrobiia	Acidimicrobiales		
11	Actinobacteria	Acidimicrobiia	Acidimicrobiales	AKIW874	
12	Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111	
13	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Iamiaceae	Iamia
14	Actinobacteria	Actinobacteria	Actinomycetales	Other	Other
15	Actinobacteria	Actinobacteria	Actinomycetales		
16	Actinobacteria	Actinobacteria	Actinomycetales	ACK-M1	
17	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Other
18	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	
19	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces
20	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Arcanobacterium
21	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	N09
22	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Trueperella

23	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	Other
24	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	
25	Actinobacteria	Actinobacteria	Actinomycetales	Beutenbergiaceae	Other
26	Actinobacteria	Actinobacteria	Actinomycetales	Beutenbergiaceae	Salana
27	Actinobacteria	Actinobacteria	Actinomycetales	Bogoriellaceae	Georgenia
28	Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	Brevibacterium
29	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Other
30	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Actinotalea
31	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomonas
32	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Demequina
33	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium
34	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Brachybacterium
35	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Dermabacter
36	Actinobacteria	Actinobacteria	Actinomycetales	Dermacoccaceae	Dermacoccus
37	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	Other
38	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	
39	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	Dietzia
40	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	Other
41	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	
42	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	Geodermatophilus
43	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	Modestobacter
44	Actinobacteria	Actinobacteria	Actinomycetales	Gordoniaceae	Gordonia
45	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Other
46	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	
47	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Janibacter
48	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Knoellia
49	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Phycicoccus
50	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Serinicoccus
51	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	Other

52	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	
53	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Other
54	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	
55	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Curtobacterium
56	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter
57	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium
58	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Mycetocola
59	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Pseudoclavibacter
60	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium
61	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Other
62	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	
63	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter
64	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Kocuria
65	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Microbispora
66	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Micrococcus
67	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Nesterenkonia
68	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Rothia
69	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Other
70	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	
71	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
72	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	
73	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus
74	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Other
75	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	
76	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium
77	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Friedmanniella
78	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides
79	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Pimelobacter
80	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Propionicimonas

81	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	Other
82	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	
83	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	Nocardiopsis
84	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiopsaceae	Thermobifida
85	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	Other
86	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	Xylanimicrobium
87	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	
89	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
90	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Tessaracoccus
91	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	
92	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Actinomycetospora
93	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Amycolatopsis
94	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Pseudonocardia
95	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Saccharomonospora
96	Actinobacteria	Actinobacteria	Actinomycetales	Ruaniaceae	Other
97	Actinobacteria	Actinobacteria	Actinomycetales	Ruaniaceae	
98	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	
99	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces
100	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	
101	Actinobacteria	Actinobacteria	Actinomycetales	Williamsiaceae	Williamsia
102	Actinobacteria	Actinobacteria	Actinomycetales	Yaniellaceae	Yaniella
103	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	
104	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium
105	Actinobacteria	Actinobacteria	Micrococcales		
106	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Other
107	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	
108	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia
109	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella
110	Actinobacteria	OPB41			

111	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	Rubrobacter
112	Actinobacteria	Thermoleophilia	Solirubrobacterales	Patulibacteraceae	
113	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	
114	Armatimonadetes	[Fimbriimonadia]	[Fimbriimonadales]	[Fimbriimonadaceae]	Fimbriimonas
115	Bacteroidetes	Bacteroidia	Bacteroidales		
116	Bacteroidetes	Bacteroidia	Bacteroidales	BA008	
117	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
118	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	5-7N15
119	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
120	Bacteroidetes	Bacteroidia	Bacteroidales	GZKB119	
121	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40	
122	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
124	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
125	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
126	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Tannerella
127	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
128	Bacteroidetes	Bacteroidia	Bacteroidales	RF16	
129	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
130	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	
131	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
132	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	CF231
133	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	[Prevotella]
134	Bacteroidetes	Bacteroidia	Bacteroidales	p-2534-18B5	
135	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	
136	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	Echinicola
137	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	
138	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Adhaeribacter
139	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter

140	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Flectobacillus
141	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter
142	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella
143	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma
144	Bacteroidetes	Flavobacteriia	Flavobacteriales		
145	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	
146	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Brumimicrobium
147	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola
148	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Other
149	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
150	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Aequorivita
151	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Capnocytophaga
152	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium
153	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides
154	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	
155	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium
156	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Cloacibacterium
157	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Ornithobacterium
158	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Wautersiella
159	Bacteroidetes	Sphingobacteriia	Sphingobacteriales		
160	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	
161	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter
162	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium
163	Bacteroidetes	[Rhodothermi]	[Rhodothermales]	Rhodothermaceae	
164	Bacteroidetes	[Rhodothermi]	[Rhodothermales]	[Balneolaceae]	
165	Bacteroidetes	[Saprospirae]	[Saprospirales]		
166	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Other
167	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
168	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Flavisolibacter

169	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Niabella
170	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Sediminibacterium
171	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Segetibacter
172	Bacteroidetes	[Saprospirae]	[Saprospirales]	Saprospiraceae	
173	Chlorobi	OPB56			
174	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	Anaerolinea
175	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
176	Chloroflexi	Anaerolineae	SBR1031	SHA-31	
177	Chloroflexi	C0119			
178	Chloroflexi	Chloroflexi	AKIW781		
179	Chloroflexi	Chloroflexi	Herpetosiphonales		
180	Chloroflexi	Chloroflexi	[Roseiflexales]	[Kouleothrixaceae]	
181	Chloroflexi	Gitt-GS-136			
182	Chloroflexi	Ktedonobacteria	B12-WMSP1		
183	Chloroflexi	TK10	AKYG885	Dolo	
184	Chloroflexi	Thermomicrobia	JG30-KF-CM45		
185	Cyanobacteria	4C0d-2	MLE1-12		
186	Cyanobacteria	4C0d-2	YS2		
187	Cyanobacteria	Chloroplast	CAB-I		
188	Cyanobacteria	Chloroplast	Stramenopiles		
189	Cyanobacteria	Chloroplast	Streptophyta		
190	Cyanobacteria	ML635J-21			
191	Elusimicrobia	Elusimicrobia	Elusimicrobiales	Elusimicrobiaceae	
192	FBP				
193	Fibrobacteres	Fibrobacteria	258ds10		
194	Firmicutes	Other	Other	Other	Other
195	Firmicutes	Bacilli	Other	Other	Other
196	Firmicutes	Bacilli	Bacillales	Other	Other
197	Firmicutes	Bacilli	Bacillales		

198	Firmicutes	Bacilli	Bacillales	Bacillaceae	Other
199	Firmicutes	Bacilli	Bacillales	Bacillaceae	
200	Firmicutes	Bacilli	Bacillales	Bacillaceae	Anoxybacillus
201	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
202	Firmicutes	Bacilli	Bacillales	Planococcaceae	Other
203	Firmicutes	Bacilli	Bacillales	Planococcaceae	
204	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
205	Firmicutes	Bacilli	Bacillales	Planococcaceae	Rummeliibacillus
206	Firmicutes	Bacilli	Bacillales	Planococcaceae	Solibacillus
207	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Other
208	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Jeotgalicoccus
209	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Salinicoccus
210	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus
211	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium
212	Firmicutes	Bacilli	Lactobacillales	Other	Other
213	Firmicutes	Bacilli	Lactobacillales		
214	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	
215	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Other
216	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus
217	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
218	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
219	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
220	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	Turicibacter
221	Firmicutes	Clostridia			
222	Firmicutes	Clostridia	Clostridiales	Other	Other
223	Firmicutes	Clostridia	Clostridiales		
224	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
225	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
226	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Other

227	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
228	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Alkaliphilus
229	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridiisalibacter
230	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
231	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Proteiniclasticum
232	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	SMB53
233	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Alkalibacter
234	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Garciella
235	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
236	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Other
237	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
238	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes
239	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
240	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio
241	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
242	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea
243	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium
244	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia
245	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Other
246	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
247	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Tepidibacter
248	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
249	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium
250	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
251	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
252	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
253	Firmicutes	Clostridia	Clostridiales	[Acidaminobacteraceae]	Guggenheimella
254	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
255	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax

256	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Mogibacterium
257	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Other
258	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	
259	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Anaerococcus
260	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Finegoldia
261	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	GW-34
262	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Helcococcus
263	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Parvimonas
264	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Peptoniphilus
265	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
266	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
267	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
268	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	ph2
269	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
270	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
271	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	Leptotrichia
272	Gemmatimonadetes	Gemm-1			
273	Gemmatimonadetes	Gemmatimonadetes			
274	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales		
275	Lentisphaerae	[Lentisphaeria]	Z20		
276	Lentisphaerae	[Lentisphaeria]	Z20	R4-45B	
277	OD1	ABY1			
278	OP11	WCHB1-64	d153		
279	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
280	Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae	
281	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
282	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	Pirellula
283	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
284	Planctomycetes	vadinHA49	PeHg47		

285	Proteobacteria	Other	Other	Other	Other
286	Proteobacteria	Alphaproteobacteria	Other	Other	Other
287	Proteobacteria	Alphaproteobacteria			
288	Proteobacteria	Alphaproteobacteria	Caulobacterales	Other	Other
289	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Other
290	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	
291	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis
292	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas
293	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
294	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana
295	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium
296	Proteobacteria	Alphaproteobacteria	Rhizobiales	Other	Other
297	Proteobacteria	Alphaproteobacteria	Rhizobiales		
298	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	
299	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Beijerinckia
300	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Other
301	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	
302	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Balneimonas
303	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
304	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Other
305	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum
306	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Other
307	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia
308	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
309	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Pedomicrobium
310	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
311	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	
312	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
313	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	

314	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus
315	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Other
316	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
317	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
318	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Other
319	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	
320	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
321	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
322	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhodobiaceae	Afifella
323	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	Hyphomonas
324	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Other
325	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
326	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Amaricoccus
327	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Jannaschia
328	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus
329	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobaca
330	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter
331	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium
332	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Other
333	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
334	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter
335	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconacetobacter
336	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Gluconobacter
337	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Roseomonas
338	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
339	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Skermanella
340	Proteobacteria	Alphaproteobacteria	Rickettsiales		
341	Proteobacteria	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	Anaplasma
342	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	

343	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Rickettsia
344	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Wolbachia
345	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	Other
346	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	
347	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	Vermamoeba
348	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Other	Other
349	Proteobacteria	Alphaproteobacteria	Sphingomonadales		
350	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Other
351	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	
352	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other
353	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
354	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Kaistobacter
355	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium
356	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium
357	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas
358	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis
359	Proteobacteria	Betaproteobacteria	Other	Other	Other
360	Proteobacteria	Betaproteobacteria			
361	Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other
362	Proteobacteria	Betaproteobacteria	Burkholderiales		
363	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Other
364	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
365	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Other
366	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	
367	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia
368	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia
369	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other
370	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
371	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax

372	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
373	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Curvibacter
374	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia
375	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Diaphorobacter
376	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
377	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Other
378	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	
379	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus
380	Proteobacteria	Betaproteobacteria	MWH-UniP1		
381	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Other
382	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	
383	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria
384	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Vogesella
385	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Other
386	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
387	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
388	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	
389	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfomicrobiaceae	Desulfomicrobium
390	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter
391	Proteobacteria	Deltaproteobacteria	GMD14H09		
392	Proteobacteria	Deltaproteobacteria	Myxococcales		
393	Proteobacteria	Deltaproteobacteria	Myxococcales	0319-6G20	
394	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	
395	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Plesiocystis
396	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
397	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Campylobacter
398	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
399	Proteobacteria	Gammaproteobacteria	Other	Other	Other
400	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Other

401	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
402	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	
403	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Ruminobacter
404	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio
405	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
406	Proteobacteria	Gammaproteobacteria	Alteromonadales	[Chromatiaceae]	Rheinheimera
407	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	Other
408	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	
409	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Other
410	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
411	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
412	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
413	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
414	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Providencia
415	Proteobacteria	Gammaproteobacteria	Legionellales		
416	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	
417	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Rickettsiella
418	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Other
419	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax
420	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Other
421	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	
422	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Nitrincola
423	Proteobacteria	Gammaproteobacteria	Pasteurellales		
424	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Other	Other
425	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	
426	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
427	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter
428	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Other
429	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	

430	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
431	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	
432	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	Other
433	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	
434	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	
435	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Nevskia
436	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Other
437	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
438	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Arenimonas
439	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Ignatzschineria
440	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
441	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Wohlfahrtiimonas
442	Spirochaetes	MVP-15	PL-11B10		
443	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
444	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
445	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
446	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
447	TM7	TM7-3	Other	Other	Other
448	TM7	TM7-3			
449	TM7	TM7-3	Blgi18		
450	Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmataceae	Acholeplasma
451	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma
452	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
453	Tenericutes	RF3	ML615J-28		
454	Verrucomicrobia	Opitutae	Puniceicoccales	Puniceicoccaceae	
455	Verrucomicrobia	Opitutae	[Cerasicoccales]	[Cerasicoccaceae]	
456	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia
457	Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	Candidatus Xiphinematobacter
458	[Thermi]	Deinococci	Deinococcales	Deinococcaceae	Deinococcus

459	[Thermi]	Deinococci	Deinococcales	Trueperaceae	B-42
460	[Thermi]	Deinococci	Thermales	Thermaceae	Thermus