Characterization of *Spirocerca lupi* in wild and domestic canids

by

Wiekolize Rothmann

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Supervisor: Dr. Pamela De Waal

To my dad

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DECLARATION

I, Wiekolize Rothmann, hereby declare that the dissertation submitted herewith for the degree Magister Scientiae at the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

Wiekolize Rothmann

July 2016

SUMMARY

Spirocerca lupi is a parasitic nematode that causes spirocercosis predominantly in domestic dogs. This disease is often fatal if not detected early before malignant transformation occurs. The nematodes may also cause serious aortic lesions which may weaken arteries and cause fatal haemorrhaging. There is currently no cure for the disease. Coprophagous dung beetles serve as intermediate hosts for the parasite. Lizards and birds serve as paratenic hosts when they consume dung beetles containing infective larvae.

Spirocerca lupi may pose a threat to wild carnivores due to the encroachment of humans with domestic dog pets near wildlife borders. There is very little information available regarding the spread of *S. lupi* among wild carnivores. In this study nematodes were collected from black backed jackals as well as several domestic dogs around the country. Corresponding haplotypes of *cox1* were identified in the black backed jackals and domestic dogs. This is a clear indication of gene flow of *S. lupi* between these two species. Phylogenetic analysis indicates that *S. lupi* from South Africa may be a cryptic species. Further analysis is still required to confirm this as only a small section of the *cox1* gene was used during the analysis. Further investigation with a larger section of the *cox1* gene as well as other genes may be required to further classify *S. lupi* in South Africa as well as other countries around the world.

To further investigate the role of *S. lupi* infection among wild carnivores a faecal DNA extraction method was designed to screen these carnivores. Many challenges were encountered since *S. lupi* egg shedding is irregular and faecal samples from various wild carnivores were not always of the correct consistency to be analysed. The eggs of *S. lupi* were also found to be very resilient and did not yield easily to disruption. Other nematodes were identified with these methods indicating that it is a potential option for other nematodes but not for *S. lupi*. In addition to these challenges, the methods tested were found to be time consuming and costly without reliable repeatability.

Several adult *S. lupi* nematodes were tested for endosymbiotic bacteria. Targeting endosymbiotic bacteria with antibiotics could be an alternative treatment for nematode infection. No endosymbiotic bacteria were detected in *S. lupi* from South Africa with the primers tested. Further analysis may yet reveal some symbiotic relationship but current results do not suggest that is a feasible option for alternative treatment.

ACKNOWLEDGEMENTS

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I would also like to acknowledge Carol Thobela-Mabaso curator of carnivores at the National Zoological Gardens (NZG) of Pretoria for coordinating the collection of faecal samples from wild carnivores. All the veterinarian practitioners around the Pretoria Metropole area for assisting with nematode collection, as well as communicating with dog owners with regard to faecal collections. You are too many to name individually but your assistance was crucial in this study. I would like to specifically acknowledge Dr. Sarah Clift at the Onderstepoort veterinary academic hospital for collecting nematodes after necropsy of dogs diagnosed with spirocercosis. Additionally I would like to acknowledge the following individuals for assisting with the collection of nematodes: Dr. Adri Steenkamp for assisting with the collection of *S. lupi* nematodes from black backed jackals; Dr. Rick Last for the adult *S. lupi* nematode samples from Durban and Prof Dawie Kok from Clinvet International for the adult *S. lupi* nematode samples from Grahamstown. Lastly I would like to acknowledge my fellow colleagues for your patience, understanding and support throughout my MSc.

ABBREVIATIONS

FF – Faecal flotation

FFH – Faecal flotation and bead homogenization

SeqS – Sequential sieving

SeqSH – Sequential sieving with bead homogenization

SS – Simplified sieving

SSH – Simplified sieving with bead homogenization

wsp – *Wolbachia* surface protein

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Chapter 1:

Literature review

INTRODUCTION

Gastrointestinal helminths are present on a global scale and many of these parasites can infect a range of different host species (WEINSTEIN and LAFFERTY 2015). Understanding of the epidemiology and ecology of Helminths shared by wild and domestic carnivores is limited largely due to the complex life cycles of these parasites. Over the years changes in ecological factors as well as human influences have contributed to the transmission of parasites between domestic and wild carnivores (OTRANTO *et al.* 2015). This occurs due to humans creating new host-parasite dynamics through introductions between these parasites and hosts. This is amplified by continued international trade and human movement. Humans also change the transmission dynamics by altering host density and thereby host-parasite contact rates as well as parasite survival (WEINSTEIN and LAFFERTY 2015).

The location and date when domestic dogs first diverged from wolves is largely disputed. There appears to be higher diversity among the domestic dogs of Southern East Asia with a date of origin ranging from 16 700 – 30 000 ya (PANG *et al.* 2009; THALMANN *et al.* 2013; WANG *et al.* 2016). One study also suggests that there are at least 51 female wolf founders as determined by mtDNA data (PANG *et al.* 2009). This corresponds to the 51 domesticated dog nematode species documented (WEINSTEIN and LAFFERTY 2015). Of the 51 domestic dog nematode species, 17 originate from dogs (Figure 1.1). From these 17 there are 14 that can be found in wildlife. This indicates that there might be parasites that have spilled over from domestic dogs to wildlife species and *vice versa* (reviewed in WEINSTEIN and LAFFERTY 2015). An example of a spillover of *Spirocerca lupi* to an introduced new species is the raccoon. Raccoons were officially released from captivity into the wild in 1934 due to a lack of food during the Second World War. As a result, raccoon populations have increased in numbers in Germany and have spread to other parts of Europe (LUTZ 1995). A study in the Warta Mouth National Park (Poland) found that raccoons have a relative high dietary preference for coprophagous insects (34%) and as a result now have a relatively high prevalence (8.8%) of *Spirocerca lupi* infection (POPIOŁEK *et al.* 2011). This is similar to a study in Japan where raccoons acquired their nematode infections from the indigenous wildlife (SATO and SUZUKI 2006).

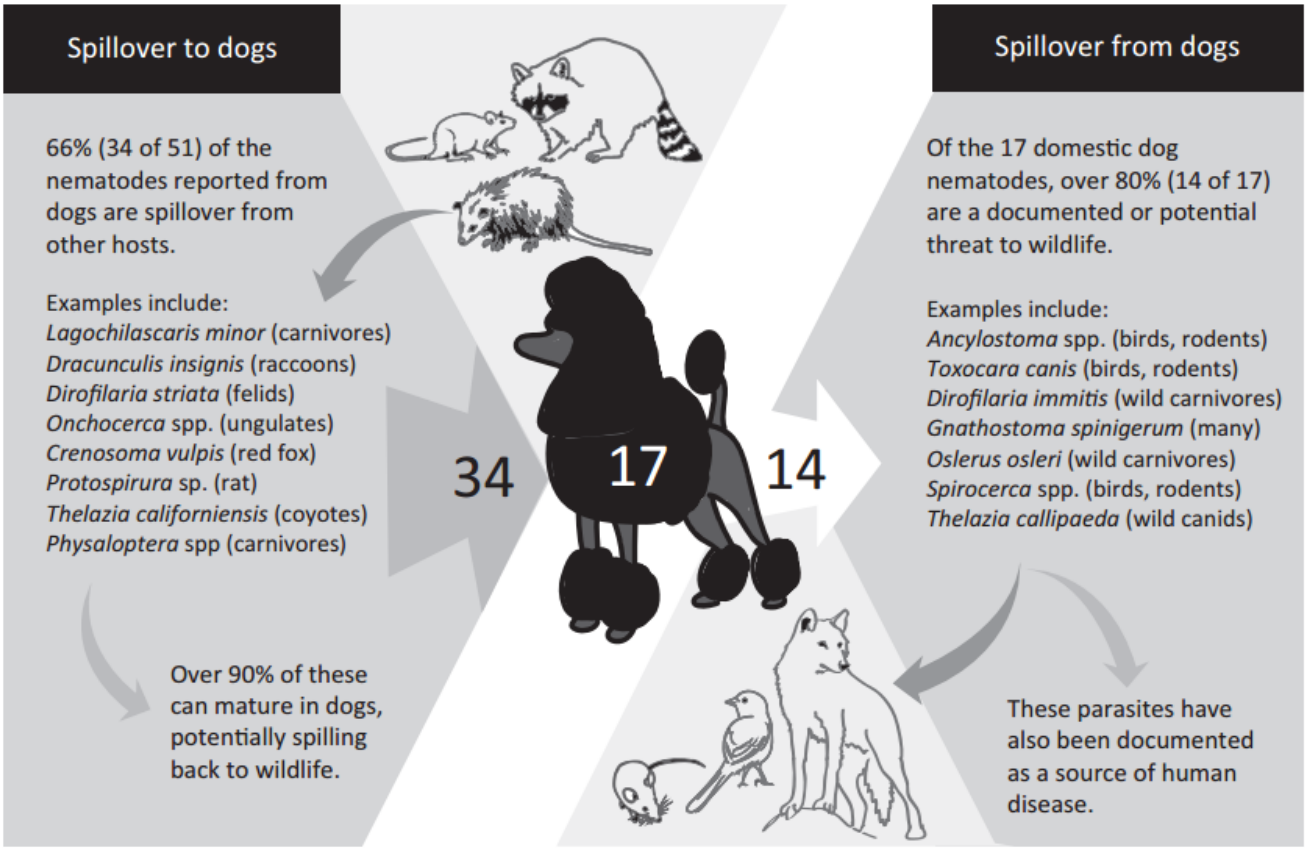


Figure 1.1: Domestic dog parasite spillover. The domestic dog is host to at least 51 parasitic nematodes, 34 (66%) of which are spilled over from wildlife hosts. Of the 17 dog parasites, 14 (80%) represent a disease risk for other wildlife (reviewed by WEINSTEIN and LAFFERTY 2015).

Spirocerca lupi

Spirocerca lupi (RUDOLPHI 1809) is a parasitic nematode that causes spirocercosis in canids. *S. lupi* nematodes have been found in many canid species but are most common in domestic dogs (BAILEY 1972). Other species that are also infected are coyotes (PENCE and MEINZER 1979), foxes (FERRANTELLI *et al.* 2009), wolves (CHOQUETTE *et al.* 1973) and jackals (ISLAM and NASHIRUDDULLAH 2000). *Spirocerca lupi* larvae were found tracking along the aorta of a young cheetah cub (MURRAY *et al.* 1964).

Spirocercosis is characterised by oesophageal nodular masses, and aortic lesions (BAILEY 1963; BAILEY 1972; VAN DER MERWE *et al.* 2008). These nodular masses may undergo malignant transformation to sarcoma (BAILEY 1963). Aortic rupture or the rupture of other major arteries may also occur, resulting

in large haematomas (BAILEY 1963; DVIR *et al.* 2001). Aberrant migration commonly causes respiratory, neurological and musculoskeletal problems (DVIR *et al.* 2001). The most common clinical signs include vomiting and regurgitation (MAZAKI-TOVI *et al.* 2002; AROCH *et al.* 2015). Other common clinical signs of spirocercosis are weight loss, anorexia and pyrexia (DVIR *et al.* 2001).

Adult *S. lupi* are red-pink worms with males ranging from 3 to 4 cm and females ranging from 6 to 7 cm (BAILEY 1972). Scanning electron micrograph images of *S. lupi* show the hexagonal mouth, papillae and amphids of the nematode (Figure 1.2) (NAEM 2004).

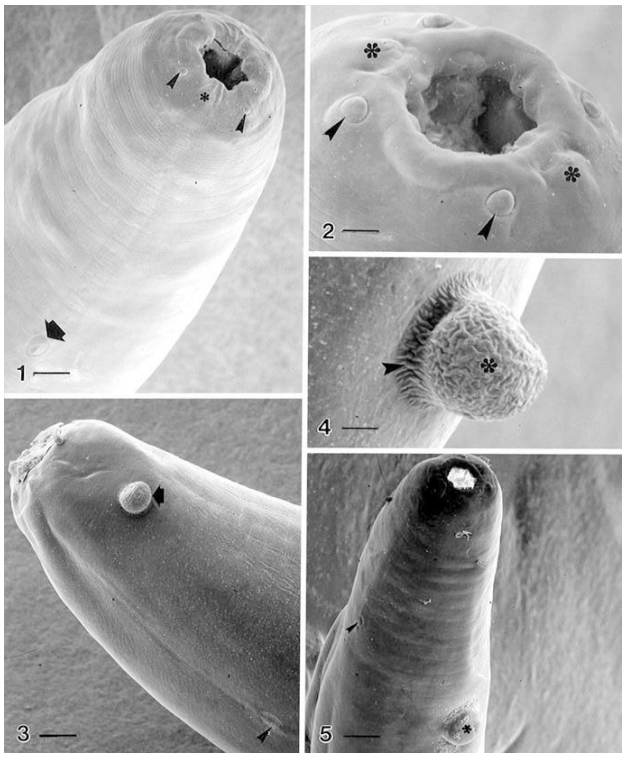


Figure 1.2: Scanning electron micrographs of *Spirocerca lupi* published by Naem (2004). **1 and 2.** Cephalic region. The hexagonal mouth with cephalic papillae (arrowheads), amphids (stars) and cervical papilla (arrow) (bar 33.3 µm). **3.** An abnormal tumour like mass (arrow, function unknown) (bar 40 µm) and **4.** Shows a higher magnification of the abnormal mass mentioned in 3 (bar 9 µm). **5.** Cervical papilla (arrowhead) and another abnormal mass (star) (bar 83.8 µm).

Coprophagous beetles serve as intermediate hosts for *S. lupi* and many smaller mammals, birds and lizards have been identified as paratenic hosts (BAILEY 1972; VAN DER MERWE *et al.* 2008). *S. lupi* infection is not limited to a single dung beetle species and some species seem to be more susceptible than others (BAILEY *et al.* 1963). This susceptibility has been attributed to the grinding efficiency of

the beetles mandibles (MILLER 1961), but beetle size may also play a role (BAILEY *et al.* 1963). In a recent study various species of dung beetle were captured and fed cattle dung with latex beads of known diameter to determine which species of dung beetle could be potential intermediate hosts for *S. lupi* (DU TOIT *et al.* 2012). Eleven out of the fourteen species tested may serve as potential intermediate host as their mouthparts allowed for beads larger than the maximum size range of the *S. lupi* eggs to pass through.

S. lupi may be transmitted by a large variety of paratenic or transport hosts. These consist mainly of birds, frogs, reptiles and small mammals (BAILEY *et al.* 1963; KRAHWINKEL JR and MCCUE 1967; VASHETKO and SIDDIKOV 1999). In green toads *S. lupi* larvae form numerous cysts in the inner organs of these amphibians. The cysts contain 1-24 nematodes in each individual toad (VASHETKO and SIDDIKOV 1999). Chickens also act as important transport hosts for *S. lupi* (BAILEY 1963; BRODEY *et al.* 1977). According to Brodey *et al*. (1977) the infective third stage larvae encyst in the crop of the chicken after the bird has ingested the infected dung beetle. Other possible paratenic hosts include millipedes of the species *Doratogonus critulatus,* which have been shown to have encysted *S. lupi* larvae after experimental infection (MUKARATIRWA *et al.* 2010). Garden lizards (*Calotes versicolor*) have also been shown to carry encysted larvae of *S. lupi* (ANATARAMAN and SEN 1966; RAVINDRAN *et al.* 2014).

Life cycle

The life cycle of *S. lupi* is shown in figure 1.3 (AUSTIN *et al.* 2013). The adult worms reside in nodular masses in the wall of the thoracic oesophagus, producing up to three million eggs per day (BAILEY 1972). The eggs, which contain L1 stage larvae (a hatching larvae is shown in Figure 1.4), are passed into the gastrointestinal tract to be shed through the faeces or sometimes by vomiting (VAN DER MERWE *et al.* 2008). Eggs are ingested by the intermediate dung beetle host and the larvae encyst within the tissues of the beetle where they develop into the infective L3 stage larvae (VAN DER MERWE *et al.* 2008). The definitive host is infected by ingestion of the paratenic or dung beetle host. Since the beetles feed within the faeces it is more likely that the dog becomes infected through coprophagia or the paratenic host (DU TOIT *et al.* 2008). The third stage larvae (L3) are liberated in the stomach and migrate through the mucosa within two hours of ingestion. Subsequently they burrow into the gastric wall and reach the serosal surface within two days. The larvae migrate within

the walls of the gastric arteries reaching the caudal thoracic aorta via the celiac artery after about ten days. They remain in the thoracic aorta until they almost reach maturity (L4) (VAN DER MERWE *et al.* 2008), then migrate through the wall of the thoracic aorta to the caudal oesophagus (SEN and ANATARAMAN 1971). The larvae burrow through the wall of the oesophagus and perforate the mucosa in order to create an opening into the alimentary canal through which the eggs can be passed. They move back into the submucosa or muscular layers to complete development (BAILEY 1972).

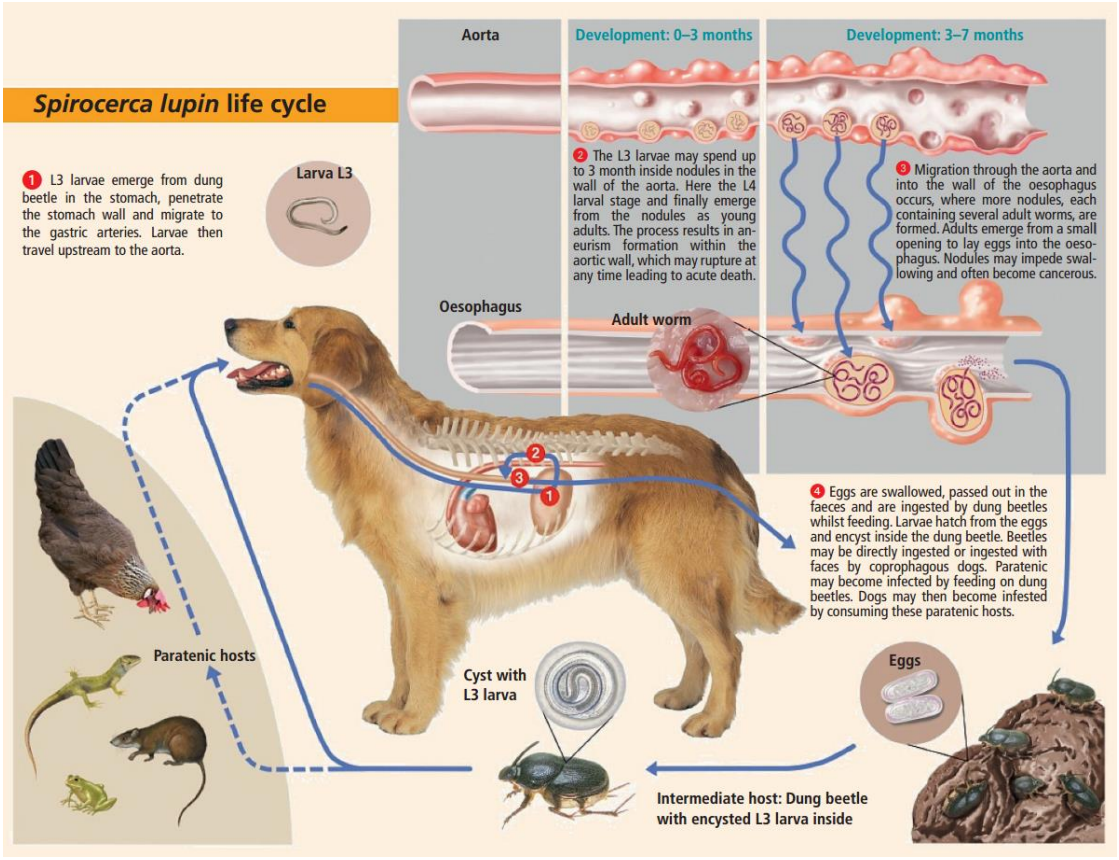


Figure 1.3: Life cycle of *Spirocerca lupi* (AUSTIN *et al.* 2013).

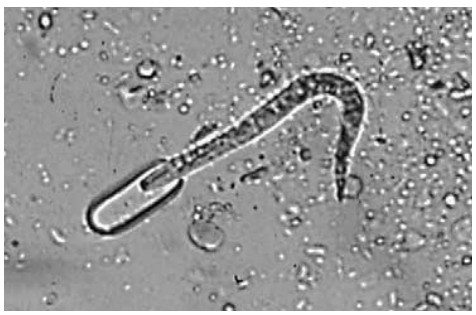


Figure 1.4: A L1 stage larvae of the *Spirocerca lupi* nematode emerging from an egg. This usually only occurs within the intermediate dung beetle host (MAZAKI-TOVI *et al.* 2002).

Incidence of *Spirocerca lupi*

S. lupi is found worldwide but is more common in warmer tropical and subtropical regions (BAILEY 1972). Since this parasite does not have a free-living stage it is independent of environmental conditions (MACPHERSON 1994). Rainfall on the other hand does play a role in the incidence and prevalence of infection as it plays a role in the life cycle of the coprophagous beetle (MACPHERSON 1994). Although, a recent case study in Israel showed only a slightly higher incidence of *S. lupi* infection with oesophageal spirocercosis during the rainy season (54.1% of the dogs infected) compared to dryer seasons (45.9% of the dogs infected) (AROCH *et al.* 2015).

Some recent reports on the incidence of *S. lupi* among dogs illustrate the widespread distribution of this parasite. In the Chittagong metropolitan area of Bangladesh the incidence of *S. lupi* among stray dogs was reported to be as high as 40% (DAS *et al.* 2011). In Northern Iran 6% (GHOLAMI *et al.* 2011), Central Mexico 4.5 % (CANTÓ *et al.* 2011), Zambia 18.7% (NONAKA *et al.* 2011) and Northeast Gabon 25.3% (DAVOUST *et al.* 2008). In South Africa in Durban and the coastal regions the Society for the Prevention of Cruelty to Animals (SPCA) reported the incidence of *S. lupi* to be 5.4% (MUKARATIRWA and SINGH 2010). In South Africa, results from a questionnaire survey indicated a possible prevalence of *Spirocerca lupi* at around 28% (LOBETTI 2000). A follow up survey indicated an apparent prevalence of 76% (LOBETTI 2014) which is similar to another study where the prevalence was found to be 71.4% (KOK *et al.* 2010). Older studies indicated a prevalence of 13% in the Free State province of South Africa (MINNAAR *et al.* 2002). The Pretoria Metropole area, in the intermediate dung beetle host, the incidence was reported to be 13.5 % in the urban areas and 2.3% in the rural areas (DU TOIT *et al.* 2008). This high prevalence in urban areas can be explained by the dense final host populations which is estimated to be 1775 dogs per square kilometre (DE WAAL *et al.* 2012). A case study in Israel also revealed a higher incidence in urban areas. The study showed that 66% of the dogs affected with oesophageal spirocercosis originated from urban areas (AROCH *et al.* 2015). There was also clear evidence that spirocercosis has spread geographically to a larger area of Israel compared to previous years (AROCH *et al.* 2015). Oesophageal spirocercosis seems to be more prevalent in larger breed dogs and retrievers (BAILEY 1963; DVIR *et al.* 2001; MAZAKI-TOVI *et al.* 2002; AROCH *et al.* 2015).

Most studies regarding spirocercosis include only the domestic dog but *S. lupi* has also been reported in many wild carnivores (BAILEY 1972; BRODEY *et al.* 1977; ENGH *et al.* 2003; MURRAY *et al.* 1964). Some of these carnivores are endemic to South Africa. Of particular interest are hyenas,

cheetahs, and jackals (table 1.1) but research regarding *S. lupi* in these animals is still lacking (MURRAY *et al.* 1964; BRODEY *et al.* 1977; ENGH *et al.* 2003). The African wild dog is susceptible to many common dog parasitic helminths such as *Ancylostoma caninum*, *Toxocara canis* and *Dipylidium caninum* (FLACKE *et al.* 2010; VAN HEERDEN *et al.* 1994), but not much data is available on its status regarding infection with *S. lupi*. The Hyenidae are more closely related to the Felids than they are to the Canids but there are records of *S. lupi* within this family (ENGH *et al.* 2003).

Another family within the wild carnivores which may also be susceptible to *S. lupi* infection is the *Herpestidae* family which includes the mongoose and the meerkat. These smaller carnivores diet includes lizards, insects and small mammals (MILLS and HES 1997), which are known paratenic hosts of *S. lupi*. Further research would be required to classify the *Herpestidae* as hosts for *S. lupi*.

Table 1.1: Larger wild carnivores with known or suspected cases of spirocercosis

Family	**Species**	**Common name**	**Description**	**Reference**
Hyaenidae	*Crocuta crocuta*	Spotted Hyena	Eggs in faeces that resemble *S. lupi*	(ENGH *et al.* 2003)
Felidae	*Acinonyx jubatus*	Cheetah	Observed cheetah with *S. lupi*	(MURRAY *et al.* 1964)
Canidae	*Canis mesomelas*	Black/Silver Backed Jackal	*S. lupi* observed in five Jackals all with aortic lesions and four with oesophageal masses.	(BRODEY *et al.* 1977)

DIAGNOSIS

Spirocercosis is usually detected through faecal flotation, endoscopy or radiographic imaging (VAN DER MERWE *et al.* 2008). Faecal flotation is not always accurate as it depends on egg shedding which is irregular and only possible once oesophageal nodules with an aperture have formed (DVIR *et al.* 2010). Radiography allows for visualization of features such as opacity near the caudal oesophagus, spondylitis and undulating of the aorta (DVIR *et al.* 2008, 2010; VAN DER MERWE *et al.* 2008). Endoscopy is more sensitive than radiography but is dependent on oesophageal nodular formation and therefore not a reliable tool for early detection (VAN DER MERWE *et al.* 2008). The detection methods have been estimated to differ in sensitivity for oesophageal mass diagnosis from 100% for endoscopy, 80% for faecal flotation and 53% for radiography (MAZAKI-TOVI *et al.* 2002).

Faecal flotation

Faecal examination (copromicroscopy) is commonly used to diagnose *S. lupi* infection, although a range of sensitivities have been reported. This range is due to irregular egg shedding and non-uniform distribution of the eggs throughout the faeces (CHRISTIE *et al.* 2011; DVIR *et al.* 2010; FOX *et al.* 1988; MARKOVICS and MEDINSKI 1996). Flotation solutions usually consist of oversaturated salt solutions that form specific gravity gradients that allow nematode eggs to float and separate from the faeces. Various salts can be used for this procedure however, a recent study revealed that of some of the different faecal examination methods, the most sensitive is a modified centrifugal faecal flotation using $NaNO_3$ at a specific gravity of 1.22 (CHRISTIE *et al.* 2011). The eggs can be viewed at X100 to X400 magnification and typically measure 20-37 µm by 11-18 µm. They are small, thick shelled and larvated (CHHABRA and SINGH 1972; SOULSBY 1982).

Radiographic imaging

Radiography is also frequently used as a diagnostic tool and more often the initial diagnosis is made by this method (VAN DER MERWE *et al.* 2008). Radiographic features that are typical of spirocercosis include opacity near the caudal oesophagus, spondylitis of the 6^{th} to 12^{th} thoracic vertebrae and undulating of the aorta (DVIR *et al.* 2008).

Endoscopy

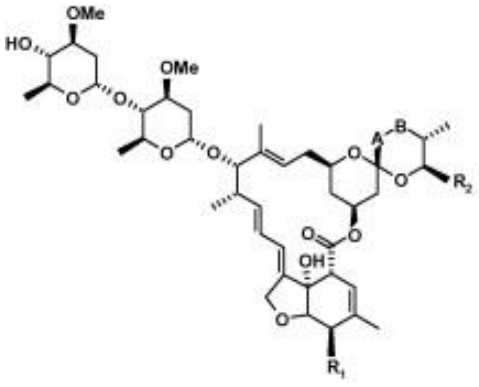
Endoscopy is currently the diagnostic test of choice since nodules in the oesophagus can be directly visualized and identified (MAZAKI-TOVI *et al.* 2002). The nodules typically appear smooth and round as they protrude into the oesophageal lumen. Neoplastic nodules usually present with roughened, ulcerated, necrotic surfaces (DVIR *et al.* 2008; VAN DER MERWE *et al.* 2008). The histological progression of the spirocercosis-associated oesophageal nodule has recently been described from early onset to formation of a sarcoma (DVIR *et al.* 2010b).

TREATMENT

There is currently no cure for this disease but it can be treated with various anthelmintic agents most of which are derivatives of avermectins. Surgical treatment has also been employed for cases where the nodular masses have undergone neoplastic transformation (VAN DER MERWE *et al.* 2008).

Anthelmintic

Naturally occurring avermectins (Figure 1.5) which consist of a group of 16-membered macrocyclic lactones that are produced through fermentation by a soil Actinomycete, *Streptomyces avermitillis*. These avermectins can be used as anthelmintics (BURG *et al.* 1979; PITTERNA *et al.* 2009). Many derivatives of these naturally occurring avermectins have been produced that possess enhanced activity against helminths (BURG *et al.* 1979; PITTERNA *et al.* 2009).



	R_1	A-B	R_2
A_1a	-OMe	-CH=CH-	*s*-butyl
A_1b	-OMe	-CH=CH-	*i*-propyl
A_2a	-OMe	$-CH_2$-CHOH-	*s*-butyl
A_2b	-OMe	$-CH_2$-CHOH-	*i*-propyl
B_1a	-OH	-CH=CH-	*s*-butyl
B_1b	-OH	-CH=CH-	*i*-propyl
B_2a	-OH	$-CH_2$-CHOH-	*s*-butyl
B_2b	-OH	$-CH_2$-CHOH-	*i*-propyl

Figure 1.5: Structures of the naturally occurring avermectins (PITTERNA *et al.* 2009).

Avermectins are anthelmintic agents, known to be effective not only in cattle or sheep but also for treating dogs with spirocercosis (EGERTON *et al.* 1979). Two avermectins, ivermectin and its derivative doramectin, have been effective in the treatment of spirocercosis (BERRY 2000; LAVY *et al.* 2002; MYLONAKIS *et al.* 2004). Doramectin (Dectomax), if administered subcutaneously at a dose of 400 µg/kg body weight, has been shown to be effective in reducing the number of eggs in the faeces of *Spirocerca lupi* infected dogs by 99.3% on day 10 after commencement of treatment. Oesophageal nodular masses significantly declined in size and number after initial treatment and were eventually completely resolved in six of the seven dogs tested (LAVY *et al.* 2002). A recent case study in Israel revealed that 20 dogs that received prophylactic treatment of 200 µg/kg subcutaneous doramectin every three months, still developed esoephageal spirocercosis despite treatment (AROCH *et al.* 2015). This might be a result of acquired nematode resistance to avermectins as was reported in studies for other nematodes although there is as of yet no such evidence regarding *S. lupi* (BOURGUINAT *et al.* 2011; GEARY *et al.* 2011; AROCH *et al.* 2015).

Ivermectin can also be effective in promoting nodule regression in naturally infected dogs. This was shown in a study where Ivermectin (600 µg/kg) was administered subcutaneously in combination with prednisolone (0.5 mg/kg) for two weeks (MYLONAKIS *et al.* 2004). Another drug, milbemycin oxime (Milbemax; Novartis Animal Health Inc.) was shown to be 79.8% effective in preventing the establishment of *S. lupi* in the oesophagus of infected dogs with only a single treatment. The drug was administered in tablet form or consisting of 12.5 mg milbemycin oxime in combination with 125 mg praziquantel of which the minimum dose was 0.5 mg milbemycin oxime/kg body weight (KOK *et al.* 2011). Another study showed that a combination of imidacloprid 10%/moxidectin 2.5% spot-on (Advocate®, Advantage® Multi Beyer Animal Health GmbH, Leverkusen Germany) can prevent canine spirocercosis (LE SUEUR *et al.* 2010). Monthly treatments of 2.5 mg of moxidectin/kg body weight has a preventative efficacy of 94.7% ($p<0.0001$). Of the 58 dogs treated only one developed spirocercosis from natural infection whereas in the untreated control group 35 of the 54 dogs developed spirocercosis as determined by endoscopy for nodules in the oesophagus. It was also stated that prevention is only possible if the dog owner adheres to a strict 24h bath free period post treatment to ensure that the active ingredient (moxidectin) can penetrate through the skin. In the dog that did develop spirocercosis, this 24h bath free period was not adhered to (LE SUEUR *et al.* 2010). A later study confirmed the efficacy of prevention to be 100% and a treatment efficacy of 98.5% against adult worms (AUSTIN *et al.* 2013). During this same study no adverse reaction towards the topically applied treatment product was observed. In July 2012, imidacloprid 10%/moxidectin

2.5% spot-on (Advocate®, Advantage® Multi Beyer) was officially licenced in South Africa for the prevention and treatment of *Spirocerca lupi* infections (AUSTIN *et al.* 2013).

Surgical

Surgery is only required when oesophageal nodules have undergone neoplastic transformation and do not regress with treatment. Oesophageal surgery may have complications that arise due to excessive tension at the suture line along with constant motion, lack of serosa, and passage of food or saliva over the suture line (VAN DER MERWE *et al.* 2008). In Israel 9 dogs underwent surgical treatment of malignant esoephageal lesions. Two dogs died during surgery whereas the remaining 7 survived to discharge (AROCH *et al.* 2015).

GENETIC DIVERSITY

Phylogeny of *Spirocerca lupi*

The complete mitochondrial genome of *S. lupi* has been sequenced (LIU *et al.* 2013). The superfamily Thelazioidea comprises of Spirurida nematodes that have hexagonal or round mouth openings that are not compressed laterally (CHABAUD and BAIN 1994). The mitochondrial gene order of *S. lupi* is identical to *Thelazia callipaeda* and *Setaria digitata*. The relationship of *S. lupi* relative to other spirurid nematodes is shown in figure 1.6 (LIU *et al.* 2013). This data is based on concatenated amino acid sequences of 12 protein coding genes from the mitochondrial sequence data analyzed through Bayesian inference.

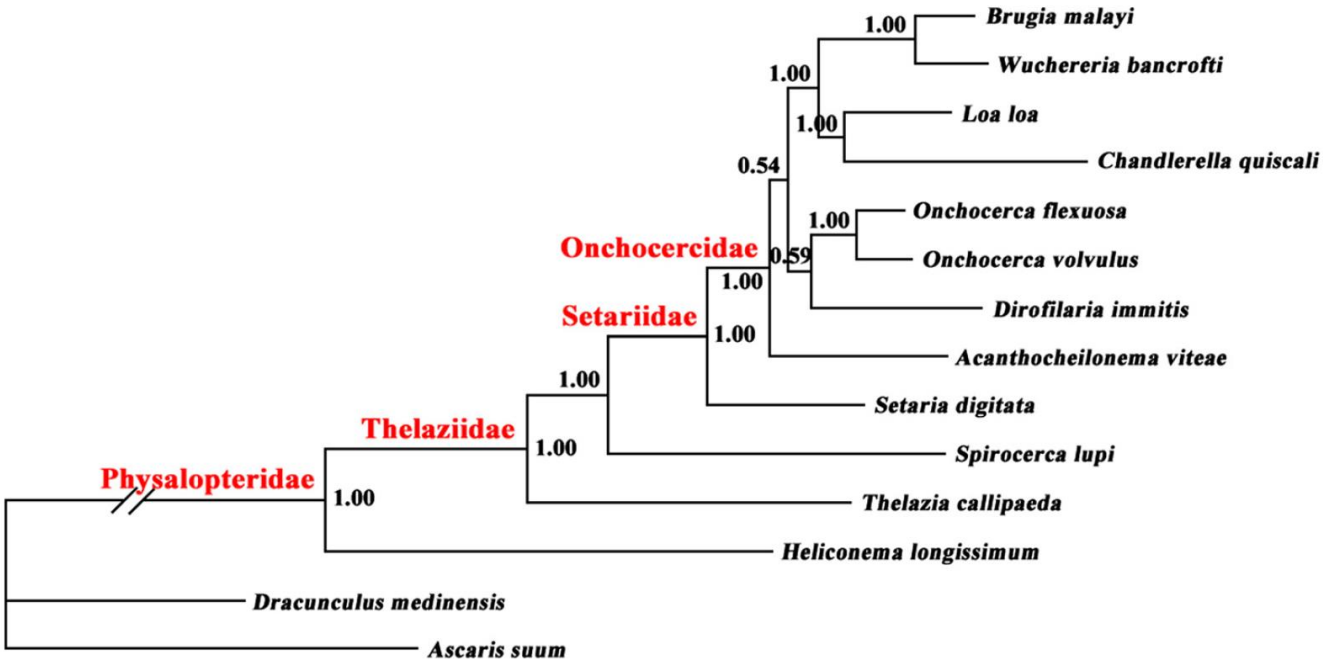


Figure 1.6: Bayesian inference of *S. lupi* with other spirurid nematodes. The posterior probability values are indicated (LIU *et al.* 2013).

Genetic variation of *Spirocerca lupi*

Two studies have looked at genetic variation in *S. lupi*, but unfortunately the results are highly contradictory. The first study used twenty *S. lupi* nematodes from different continents namely Africa, Asia and Europe (TRAVERSA *et al.* 2007). Low nucleotide variation was found within specimens from Europe or those from Africa and Asia and only two haplotypes were identified. The second study was performed in South Africa in the Tshwane Metropolitan region and compared the genetic variation of *S. lupi* by looking at *cox1* sequences obtained from nematodes isolated from twenty dogs from which each contributed three worms (DE WAAL *et al.* 2012). Eleven haplotypes were identified that could be divided into two haplogroups. Three quarters of this variation was between worms in the same host whereas the rest is explained by differences between infrapopulations. They concluded that the high within-host genetic variation was due to the high prevalence of *S. lupi* within the intermediate dung beetle host. This was due to the nematode being densely populated within the final host allowing for higher levels of amalgamation of infrapopulations.

WOLBACHIA

Recent studies on parasitic nematodes includes the search for endosymbiotic bacterium. *Wolbachia* was first discovered in filarial nematodes during the 1970s (KOZEK 1977; KOZEK and MARROQUIN 1977; VINCENT *et al.* 1975). This bacterium exhibits a symbiotic association with all filarial nematodes (WERREN *et al.* 2008). *Wolbachia*, which is cytoplasmically inherited, is commonly found in the reproductive tissue of a variety of arthropods as well as some nematodes (FOSTER *et al.* 2005; O'NEILL *et al.* 1992; ROUSSET *et al.* 1992; SIRONI *et al.* 1995; WERREN 1997; WERREN *et al.* 1995). The bacterium alters the reproductive mechanisms of the host in order to favour the development of females to promote maternal inheritance (WERREN *et al.* 2008; WERREN *et al.* 1995). Some of the changes that these bacteria induce in arthropods include cytoplasmic incompatibility (BREEUWER and WERREN 1990; O'NEILL and KARR 1990), parthenogenesis (STOUTHAMER *et al.* 1993), and feminization (ROUSSET *et al.* 1992). There is also evidence for lateral gene transfer from *Wolbachia* to the host organism (AIKAWA *et al.* 2009).

The symbiotic association between filarial nematodes and *Wolbachia* has been exploited as a new method to treat various diseases, such as lymphatic filariasis (elephantiasis), which is caused by the nematodes *Wuchereria bancrofti* and *Brugia malayi,* as well as onchocerciasis (river blindness) caused by *Onchocerca volvulus* (TAYLOR *et al.* 2010). Anti-*Wolbachia* therapy is safer and delivers superior therapeutic outcomes compared to the standard anti-filarial treatments (TAYLOR *et al.* 2010). The depletion of *Wolbachia* in *Brugia malayi* caused rapid and sustained cell apoptosis of reproductive cells, developing embryos, microfilariae and developing larvae (LANDMANN *et al.* 2011). This has also been shown in previous studies where tetracycline treatment of infected jirds (rodent) (1.2% in drinking water) showed clear embryo degeneration in *Brugia pahangi* and *Dirofilaria immitis* (BANDI *et al.* 1999). Oxytetracycline has also been tested *in vivo* against *Onchocerca ochengi* where the data showed that oxytetracycline has excellent macrofilaricidal activity since the worm burden of all treated cattle was eliminated (LANGWORTHY *et al.* 2000).

Detection of *Wolbachia*

Wolbachia is usually detected through various PCR methods. Protein coding genes that are commonly used include the *Wolbachia* surface protein (*wsp*) and the cell division protein, FtsZ. The 16S rRNA gene is also commonly used to detect *Wolbachia* (SIMOES *et al.* 2011). The *wsp* gene

evolves at a faster rate compared to other genes, therefore creating highly variable sequences that can be used to resolve the phylogenetic relationships of different *Wolbachia* strains (ZHOU *et al.* 1998). Other detection techniques include immunohistochemistry whereby *Wolbachia* can be detected in the gonads of its host, as well as non-reproductive tissue where it may also be found (DOBSON *et al.* 1999; KRAMER *et al.* 2003).

Wolbachia in nematodes

Data indicate that infection of *Wolbachia* is widespread among the filarial nematodes (BANDI *et al.* 1998). *Wolbachia* species have been grouped into eight supergroups (lineage A-H) (CASIRAGHI *et al.* 2005). Figure 1.7 shows an image from Werren *et al.* (2008) indicating the various supergroups of *Wolbachia*. *Wolbachia* in the arthropods are divided into the A and B *Wolbachia* lineages (WERREN *et al.* 1995) whereas for filarial nematodes group under the C and D lineages (BANDI *et al.* 1998). Although they fall within a distinct separate lineage the *Wolbachia* in filarial nematodes are more closely related to *Wolbachia* of arthropods than any other bacterium for which the 16S rDNA or *ftsZ* gene sequences are available (BANDI *et al.* 1998).

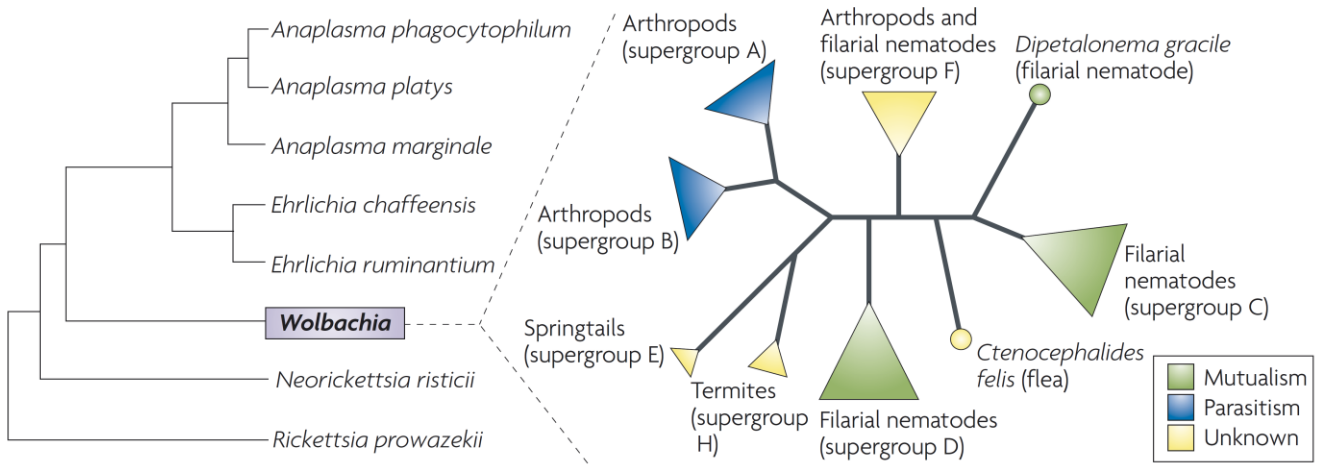


Figure 1.7: Phylogeny of *Wolbachia* showing an unrooted phylogenetic tree of the main supergroups of Wolbachia (WERREN *et al.* 2008).

AIMS AND OBJECTIVES

The domestic dog may pose a threat to wildlife carnivores as it can host various parasitic nematodes that may spill over to the wild populations. The high density of domestic dogs in urban areas also provides ideal conditions for gastrointestinal parasites such as *Spirocerca lupi*. The aim of this study is to determine if there is gene flow of *Spirocerca lupi* between domestic dogs and wild carnivore species and to find a way to safely screen wild carnivores for *S. lupi*.

The first objective of this study was to determine the genetic diversity between *S. lupi* in dogs and black backed jackals by comparing different *cox1* haplotypes. Additionally sequences of *cox1* from *S. lupi* were obtained from different regions around South Africa as well as sequences from other countries. These sequences will be used to determine the amount of diversity between regions within South Africa and between South Africa and other countries.

Another objective was to develop a method by which *S. lupi* DNA can be successfully extracted from wild carnivore faeces for non-invasive screening purposes. We can thereby determine the extent of *S. lupi* infection among these animals.

A final objective was to determine if *S. lupi* contains some bacterial endosymbiont that can be used as alternative target for treatment. A recent study showed that *S. lupi* does not contain the *Wolbachia* endosymbiont but it may harbor a novel bacterial endosymbiont that is closely related to *Comamonas* species of the beta-proteobacteria (GOTTLIEB *et al.* 2012). This discovery might open new avenues for drug treatment of spirocercosis in the domestic dog.

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Chapter 2:

Genetic variation of *Spirocerca lupi* in South African dogs and jackals from different regions

ABSTRACT

Spirocerca lupi is a parasitic nematode found in domestic dogs as well as some wild carnivores. *S. lupi* has high within host genetic variation and there seems to be a general increase in diversity when looking at various *cox1* haplotypes across South Africa. A previous study found eleven different *cox1* alleles in the Tshwane Metropole (Pretoria) area. The current study indicates sixteen haplotypes from four different regions in South Africa where thirteen of these haplotypes are novel alleles. Corresponding haplotypes are found in the domestic dog and black backed jackals indicating gene flow between these species. This might pose a threat to other wild carnivores such as the endangered African wild dog. The increase in genetic diversity might correlate with the increase in cases of spirocercosis as well as an increase in resistance to treatment. In Denmark a potential cryptic species of *S. lupi* was recently discovered in the red fox. Comparing different regions around the world indicate that South African *S. lupi* may also be a separate cryptic species.

INTRODUCTION

Spirocerca lupi is a parasitic Spirurid nematode that causes aortic lesions and esophageal nodular masses in domestic as well as some wild canids (BAILEY 1972; VAN DER MERWE *et al.* 2008). *S. lupi* is found globally in tropical and subtropical regions and may be fatal to its host (VAN DER MERWE *et al.* 2008). Coprophagous beetles serve as intermediate hosts *for S. lupi*. In the Tshwane (Pretoria) Metropole there were significant differences in the incidence of *S. lupi* among dung beetles found in rural (2.3 %), urban (13.5 %) and peri-urban (0 %) areas (DU TOIT *et al.* 2008). Birds and rodents may serve as additional paratenic hosts for the L1 stage infective larvae (BAILEY 1972; VAN DER MERWE *et al.* 2008). *Spirocerca lupi* larvae have also been identified in garden lizards (*Calotes versicolor*) (RAVINDRAN *et al.* 2014) as well as in green toads in Uzbekistan (VASHETKO and SIDDIKOV 1999).

A case study in Israel at the Hebrew University Veterinary Teaching Hospital showed a 4-fold increase (22.2 average annual cases) of oesophageal spirocercosis over a 6 year period (2004 – 2009) compared to the previous data (5.6 annual cases) collected during 1991 and 1999 (AROCH *et al.* 2015). This could be due to the increased awareness of Israeli dog owners as well as veterinarians in the region leading to more dogs being diagnosed yearly (AROCH *et al.* 2015). A recent study in South Africa indicates that the apparent prevalence of *S. lupi* is 76% as determined through a nationwide survey (LOBETTI 2014). The highest prevalence was determined to be in Gauteng and KwaZulu-Natal provinces. This survey also indicated that there was an increase in the prevalence of *S. lupi* between 1998 and 2012. Some of the major changes between the 1998 and 2012 surveys indicate that there is an increase in the efficacy of *S. lupi* treatment and a decrease in the overall mortality rate in domestic dogs (LOBETTI 2014).

Mitochondrial DNA (mtDNA) is useful for phylogenetic analysis as it is maternally inherited, it has a rapid rate of divergence and no recombination. This allows a researcher to distinguish between closely related species (AVISE *et al.* 1987; MORITZ 1994). A study in the Tshwane Metropole (Pretoria) area indicated that the highest diversity was found within the domestic dog host (76%) compared to the diversity among hosts (DE WAAL *et al.* 2012). In this same study, eleven haplotypes were found among 60 adult nematodes. In Denmark, a genetically distinct isolate of *S. lupi* was found in the red fox (*Vulpes vulpes*) (AL-SABI *et al.* 2014).

This study utilizes the mitochondrial *cox1* gene of *S. lupi* in order to determine the diversity of *S. lupi* in South Africa. Nematodes were collected from four different regions in South Africa from

domestic dogs as well as three black backed jackals. Genetic variation between these regions should give some insight into the dispersion *of S. lupi* around South Africa. A comparison can also be made with other countries as data is now available from Europe, Israel and China.

MATERIALS AND METHODS

Nematodes

A total of 57 adult *S. lupi* nematodes were obtained from various regions of South Africa. Of these nematodes 9 were from dogs from Durban in Kwa-Zulu Natal, 12 from Grahamstown in the Eastern Cape, and 28 from the Tshwane Metropole in the Gauteng Province. Seven *S. lupi* nematode larvae were also obtained from black backed jackals from Kimberley in the Northern Cape. The nematode larvae were extracted post mortally from the dogs as well as jackals. All nematodes were stored in 70% ethanol at 4 °C until DNA extraction was performed.

DNA extraction

The nematodes were divided into three sections. Two sections were stored in 70% ethanol at 4°C. The remaining section was washed three times in 96% ethanol and transferred to a clean eppendorf tube. The worms were crushed with liquid nitrogen. DNA extraction was performed with the DNeasy® blood and tissue kit (Qiagen) following the protocol for purification of total DNA from animal tissues.

Polymerase chain reaction and colony polymerase chain reaction

Primers JB3 (5'- TTTTTTGGGCATCCTGAGGTTTAT) and JB4.5 (5'- TAAAGAAAGAACATAATGAAAATG) (BOWLES *et al.* 1992) were used to amplify the cytochrome c oxidase subunit 1 in *S. lupi*. These primers have previously been tested on *S. lupi* and were shown to amplify a 440bp region of *cox1* (DE WAAL *et al.* 2012). The PCR reaction consisted of the following reagents: 100 pmol of each primer, 2.5 mM of each dNTP, 1x Ex Taq buffer (containing 20 mM $MgCl_2$), 1 unit TaKaRa Ex Taq (Takara biotechnology) and approximately 100 ng of template DNA. The reaction was made up to a final volume of 50 µl with ultra-high quality water. The reaction was set to an initial 5 min

denaturation at 95°C followed by 30 cycles of 95°C for 1min, 58°C for 1min, 72°C for 1min. A final elongation step of 7 min at 72°C was included to ensure that the DNA was fully elongated.

Colony PCR was implemented after cloning before miniprep purification to determine if the correct inserts were present. The commercially available primers pUCM13 forward and reverse primers (Promega) were used for this PCR. The PCR reaction consisted of 100 pmol of each primer, 2.5 mM of each dNTP, 1x GoTaq® flexi green buffer, 2 mM $MgCl_2$ and 1 unit GoTaq® DNA Polymerase. The template was a few cells from the selected cloned colony. The reaction mixture was made up to a final volume of 50 µl with ultra-high quality water. An annealing temperature of 50°C was used with conditions similar to that for the PCR above except the initial denaturation was performed for 10 min at 96°C in order to disrupt the bacterial cells.

Agarose Gel electrophoresis

Agarose electrophoresis gels were made from 1% agarose, 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and ethidium bromide at 25 ug/35 ml of agarose gel. All analyses were performed at 100V for 40 min to ensure adequate separation. Gels were examined under a 70% fluorescent UV light and the correct sized band was excised from the agarose gel and the DNA purified with the High pure PCR product purification kit (Roche).

Cloning

Cloning was performed with the StrataClone® PCR cloning kit (Agilent technologies) using StrataClone® SoloPack competent cells (Agilent technologies) according to the manufacturers instructions. Ligation reactions were incubated overnight at 4°C to ensure adequate ligation before transformation was performed. Cells were plated on Luria-Bertani medium agar plates supplemented with 100 µg/ml ampicillin, 50 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Blue-white screening was implemented. Selected colonies were grown overnight at 37˚C in liquid cultures consisting of liquid Luria-Bertani medium supplemented with 100 µg/ml ampicillin. From these cultures small preparations of plasmid DNA were prepared using the Wizard® plus V Minipreps DNA Purification System (Promega). The centrifugation protocol was followed as described by the manufacturers.

Sequencing

Automated sequencing reactions were prepared consisting of 1x Big Dye dilution buffer, BigDye® Terminator v3.1 and 3.2 pmol of the commercially available pUCM13F primer (Promega). Approximately 600 ng of plasmid DNA was used in the sequencing reaction. The final volume was made up to 10 µl with Ultra High quality water. The sequencing reaction commenced with an initial 1 min denaturation step at 96˚C followed by 25 cycles of 95˚C for 1 sec, 50˚C for 20 sec and 60˚C for 4 min. The sequences were purified after the cycle reaction by placing the entire 10 µl reaction in 50 µl absolute ethanol with 3 µl 3 M sodium acetate at pH4.6. The samples were incubated overnight at 4˚C to allow for DNA precipitation. The samples were centrifuged for 30 min at 14000 rpm and the pellet washed twice in 250 µl 70% ethanol. The samples were centrifuged for 5 min at 14000 rpm between each wash step. The pellet was dried overnight at 4˚C and processed at the African Center for Gene Technologies (ACGT) DNA sequencing facility at the University Of Pretoria using the ABI 3100 or ABI 3500 systems.

Data analysis

Sequences were manually edited with CLC Genomics Workbench 8.0.3 (CLC Bio-Qiagen, Aarhus, Denmark) and BioEdit version 7.2 (HALL 1999). Sequence data was compared to the data on the National Center for Biotechnology Information (NCBI) using the nucleotide basic local alignment search tool (nBLAST) (ALTSCHUL *et al.* 1990). Haplotypes (accession HQ674751-HQ674761) of *cox1* for S. *lupi* from a previous study were downloaded from the NCBI database in order to compare with current data (DE WAAL *et al.* 2012). In addition *cox1* sequences of *S. lupi* from Denmark, China, Israel, Italy, Iran and Austria were downloaded from the NCBI database and only the overlapping region (approximately 156 bp) was used for accurate comparison. *Thelazia callipaeda* and *Gongylonema pulchrum cox1* sequences were downloaded as outgroups.

DNAsp (ROZAS 2009) was used to create haplotype data files. Amino acid translations were obtained through DNAsp using the genetic code setting for *Drosophila* as this corresponds to the genetic code of *S. lupi* previously described (LIU *et al.* 2013). Arlequin 3.5 (EXCOFFIER and LISCHER 2010) was used to perform an AMOVA in order to test three levels of population subdivision. The subdivisions consisted of the population within the host, the variance between different hosts

within a region and the variance between regions. Statistics were calculated with R3.2.1 (R CORE TEAM 2013).

Phylogenetic trees were inferred by Hasegawa-Kishino-Yano model with gamma distributed rates and patterns conducted in MEGA6 with a 1000 bootstap replications (TAMURA *et al.* 2013). Pairwise distance of *cox1* was also calculated with MEGA6. Haplotype networks were drawn with the software Network 4.613 (BANDELT *et al.* 1999) using the median joining method. Bayesian inference (BI) was performed with MrBayes 3.2 using the Monte Carlo Markov Chain (MCMC) method (HUELSENBECK and RONQUIST 2001; RONQUIST *et al.* 2012) and a phylogenetic tree was constructed using FigTree 1.4 (http://tree.bio.ed.ac.uk/).

RESULTS AND DISCUSSION

In this study, *S. lupi* nematodes were collected from various regions within South Africa from domestic dogs as well as black-backed jackal. The mitochondrial *cox1* gene was used in order to determine the genetic diversity within these regions. This information should give some insight into the distribution and current diversity of *S. lupi* in this country.

Polymerase chain reaction

PCR was performed with primers that amplify a region of *cox1* of *S. lupi*. These primers have previously been tested on *S. lupi* adult nematode DNA and successfully amplified a 440 bp region of *S. lupi cox1* (DE WAAL *et al.* 2012). All the samples from this study which consists of larvae as well as adult nematodes yielded an amplification product in the order of 440 bp. The *cox1* amplified from nematode larvae obtained from jackal is shown in figure 2.1. The amplicons were excised from agarose gels and purified with the High pure PCR product purification kit (Roche) and cloned.

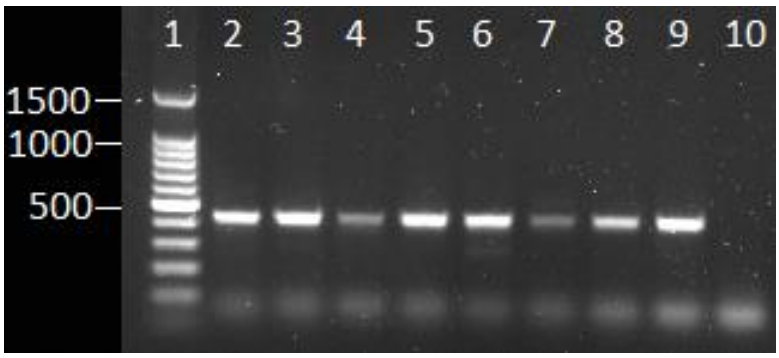


Figure 2.1: Agarose gel electrophoresis analysis showing the product of a PCR with DNA from nematode larvae obtained from jackal. Lane 1 is a 100 bp ladder, lane 2-9 are the seven nematode larvae samples from Jackal and lane 10 is the negative control.

Blue white screening was performed on the colonies obtained after cloning. White colonies were selected for each cloned nematode DNA sample and liquid cultures were prepared. Recombinant white colonies were screened for inserts using the pUCM13 forward and reverse primer in a colony PCR. The results for one such PCR is shown in figure 2.2. Liquid cultures that showed successful amplification with the colony PCR, with the correct number of base pairs, were used in plasmid purification using the Wizard plus V Minipreps DNA Purification System (Promega). The plasmid inserts were sequenced using the pUCM13 primers.

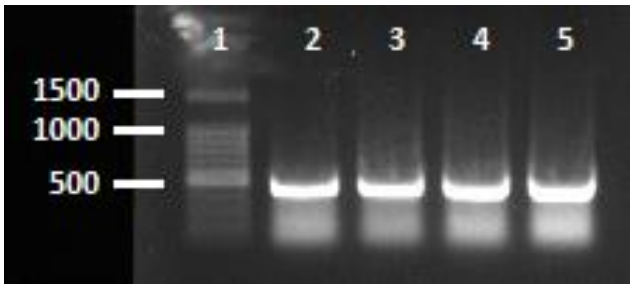


Figure 2.2: Agarose gel electrophoresis of the products of a colony PCR. Lane 1 is the 100 bp ladder. Lane 2-5 represents the different colonies of bacteria that were amplified to determine if the correct insert was present before mini-preparation of DNA samples.

Sequencing results

Sequences with Phred quality scores above 40 were used. Sequences with lower quality scores where there were errors in the 440 bp segment were re-sequenced and the sequences aligned to ensure that the correct sequence was obtained. Figure 2.3 shows a chromatogram from one of the sequences. The sequences were cut down to 346 bp which is the same length with the same base positions as previously published samples for *S. lupi cox1* (DE WAAL *et al.* 2012). This facilitated accurate comparison of the results obtained in this study with previous published data.

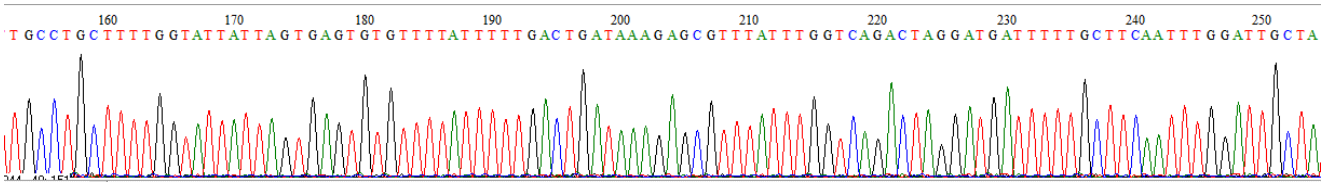


Figure 2.3: Sequencing chromatogram of a segment of sequence from *cox1*.

Of the 57 nematode samples only 56 sequences were used since one sequence contained a deletion that resulted in a frameshift mutation ending in a premature stop codon. This deletion may be due to an error obtained during PCR amplification, but this is unlikely since *TaKaRa Ex Taq* DNA polymerase contain proofreading activity. The deletion might also be due to heteroplasmy, where only a certain number of the mitochondrial genome copies contain the deletion. Since the cause of the deletion is unclear the sample was removed in order to ensure more accurate results from the data set. A list of all the samples, their nBLAST results and their assigned haplotypes can be seen in appendix 1. The samples are arranged according to their assigned haplotypes.

Analysis of molecular variance

AMOVA was performed using Arlequin 3.5 (EXCOFFIER and LISCHER 2010) to determine the genetic variation in *cox1* between the *S. lupi* nematodes obtained from different regions of South Africa. The AMOVA results were similar to previous results indicating that the most genetic variation is between nematodes within the host (68.63%) as can be observed in table 2.1. The second highest variation was among populations within the regions. Very little variation was found between the different regions.

Table 2.1: AMOVA of the *S. lupi* adult nematodes.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among regions	3	19.223	0.23434 Va	7.83
Among populations within regions	28	89.073	0.70414 Vb	23.54
Within host	22	45.167	2.05303 Vc	68.63
Total	53	153.463	2.99151	100

Population genetic structure

The genetic structure and diversity of *cox1* of *S. lupi* across South Africa should give some insight into the dispersion, diversity and gene flow of the parasite around the country. Sixteen haplotypes were identified among the 56 sequences of *cox1* from different districts in South Africa with a haplotype diversity of 0.8234. This high diversity among the haplotypes indicates that there is a high probability of randomly sampling two different alleles. These sequences contained a total of 29 variable sites where alignment gaps were not considered. The nucleotide diversity per site was (π) 0.01633. The low nucleotide diversity indicates low polymorphism in the gene sequences. These 56 sequences all consisted of 346 nucleotides that could be translated into open reading frames (figure 2.4).

```
[                 10        20        30        40        50        60        70        80        90       100       110
[                  *         *         *         *         *         *         *         *         *         *         *
Haplotype1   IISECVLFLTDKERLFGQTSMIFASIWIAILGTSVWGHHMYTAGLDIDTRTYFSAATMIIAIPSAVKVFNWLGTFFGSRQKIQPLWCWTYSFIFLFTLGGLSGIVLSTASLDIVL
Haplotype4   ....................................................................................................................
Haplotype9   ....................................................................................................................
Haplotype12  ....................................................................................................................
Haplotype13  ....................................................................................................................
Haplotype14  ....................................................................................................................
Haplotype15  ..G.................................................................................................................
Haplotype16  ....................................................................................................................
Haplotype17  ...................G................................................................................................
Haplotype18  ....................................................................................................................
Haplotype19  ..............................................................................E.........................................
Haplotype20  ....................................................................................................................
Haplotype21  ......................V.............................................................................................
Haplotype22  ...................T................................................................................................
Haplotype23  ....................................................................................................................
Haplotype24  ....R...............................................................................................................
```

Figure 2.4: Variation in amino acid sequence of the different haplotypes.

A maximum likelihood tree was constructed using the software MEGA6. This was performed in order to determine if there was some variation among the sequences of *S. lupi* samples from dogs and jackal. *Thelacia callipaeda* and *Gongylonema pulchrum* was used as outgroups. The maximum likelihood tree can be split into two haplogroups as seen in figure 2.5.

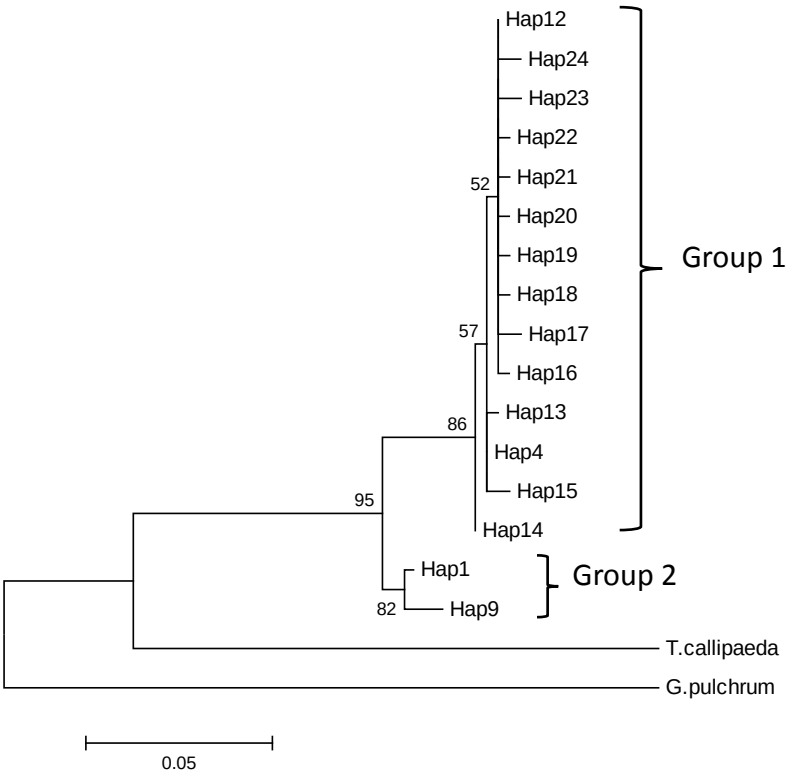


Figure 2.5: Maximum likelihood tree of *cox1* from *S. lupi* nematodes showing the different haplotypes. Bootstrap values are shown at each node.

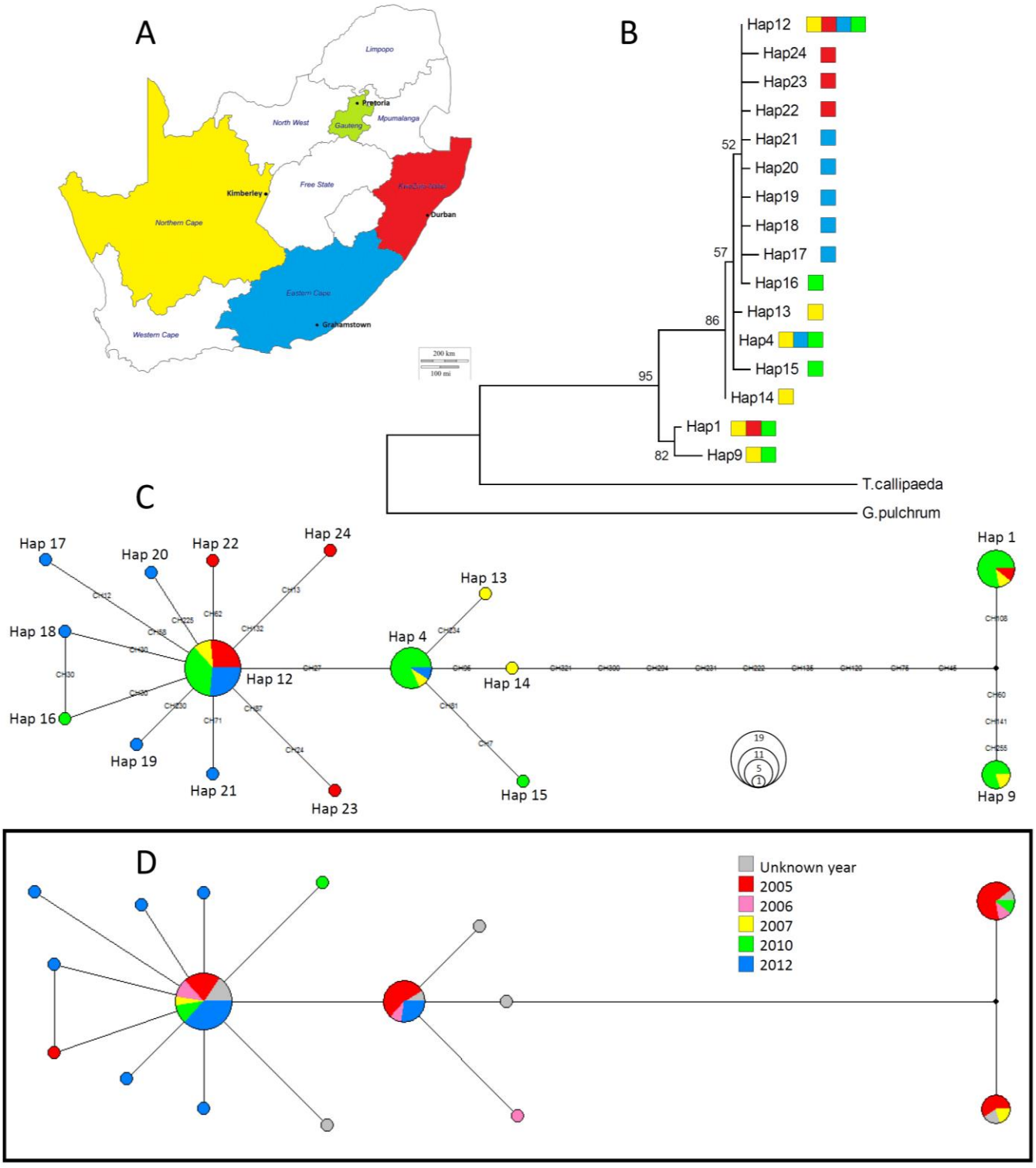
A haplotype network was constructed using the software network. This was performed in order to obtain a broader view of the diversity between different regions in South Africa. The haplotype network is shown in figure 2.6 along with a map of South Africa and the before mentioned maximum likelihood tree with the various haplotypes. Another haplotype network was constructed (figure 2.6D) to show the collection year of each sample. From the haplotype network it's clear that haplotype 12 is the most common haplotype and it is also present in all four regions. This could be the founding haplotype or the one closest to the first introduced *Spirocerca lupi* infection. Haplotypes 9 and 1 seem to be older and more common in Pretoria than the other regions. This could be because they were sampled first and because Pretoria has a relatively larger sample size compared to the other regions.

Most of the newly sampled haplotypes are unique to their region. These might be newly evolved and have not yet spread to other regions. The samples from the Jackal (yellow) seem to share most of the haplotypes with the other regions, except for haplotype 13 and 14 which are unique to the jackal population. Since the samples from Kimberley are only from the black-backed jackals and not

any domestic dogs it is unknown if the same haplotypes would be found in the dog population in that region.

Sequences of *Spirocerca lupi cox1* were obtained from NCBI Genbank from regions in Europe, Asia and the Middle East. There was only a 156 bp overlap between these sequences and our data, therefore all the sequences were shortened for the analysis to be accurate and consistent. The samples from Denmark were obtained from the red fox (*Vulpes vulpes*). A maximum likelihood tree (figure 2.7) and a Bayesian Inference (figure 2.8) indicate that the sequences from South Africa differ from those of other continents which is similar to data that was found for the red foxes from Denmark (AL-SABI *et al.* 2014). The maximum likelihood and Bayesian inference suggests that there are three groups of *S. lupi*. The maximum likelihood and Bayesian inference have strong bootstrap support for the separation of groups A and B from C with weaker support for the separation of A from B. A similar case can be seen in Al-Sabi's (2014) Neighbor-Joining and Bayesian Inference phylogenetic trees where the separation between the Denmark and South African samples have slightly lower bootstrap support.

Figure 2.6 (on following page): Haplotype diversity of *Spirocerca lupi* for cytochrome c oxidase subunit 1 **A:** A map of South Africa indicating the nematode origin. **B:** Maximum likelihood tree showing the various haplotypes of *S. lupi*. The coloured blocks indicate in which regions samples were found. **C:** Haplotype network of the *S. lupi* nematodes from domestic dogs as well as the black-backed jackals. The circle sizes are relative to the frequency of the specific haplotypes. The coloured pie charts indicate the regions where the haplotypes were derived from. **D:** Haplotype network showing the timeline when the various haplotypes were collected.



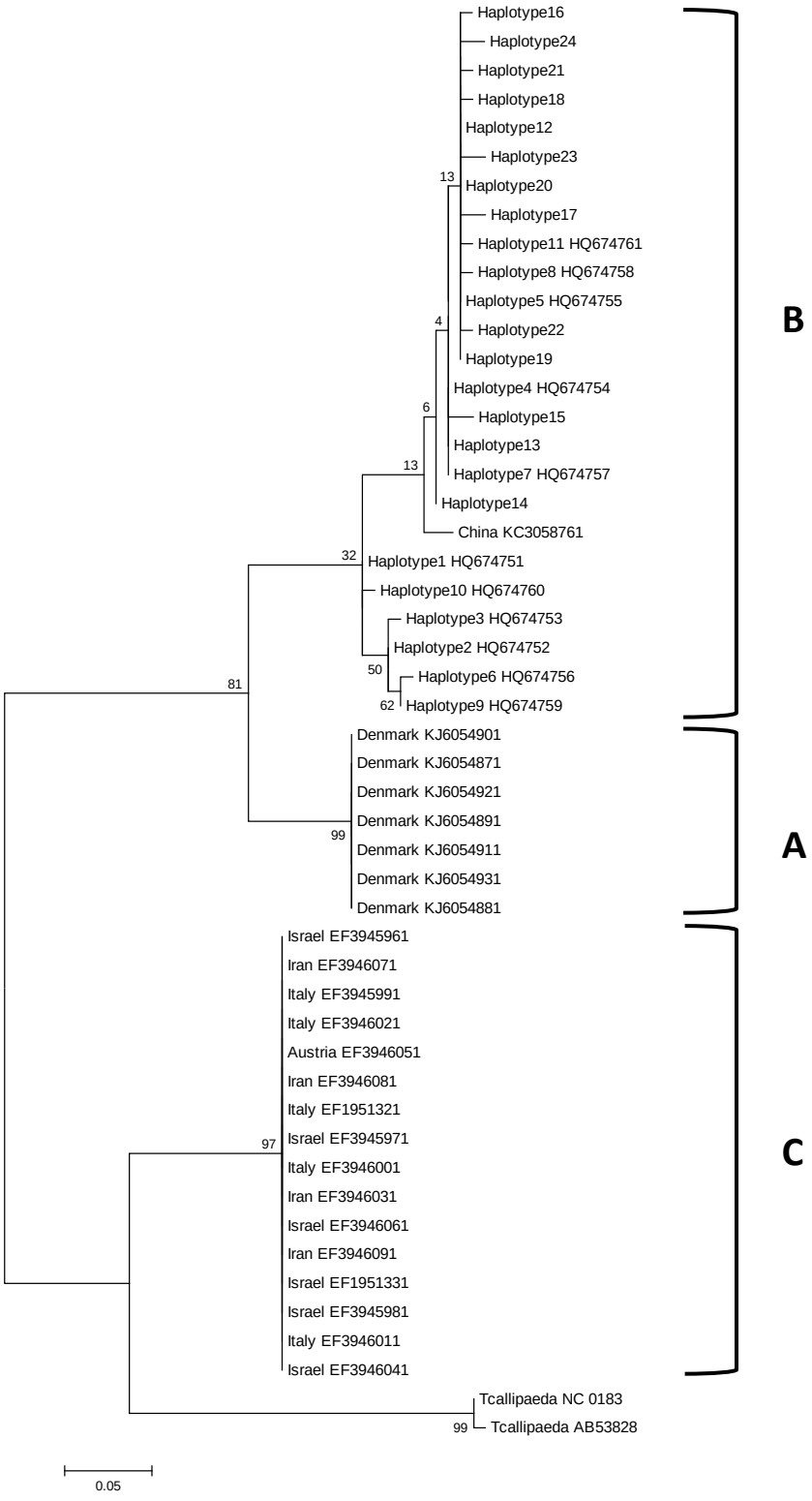


Figure 2.7: Maximum likelihood tree with 156 bp sequences of *S. lupi cox1* from different continents. Bootstrap values are indicated at the nodes. The scale bar indicates distance. Three groups indicated by A, B and C.

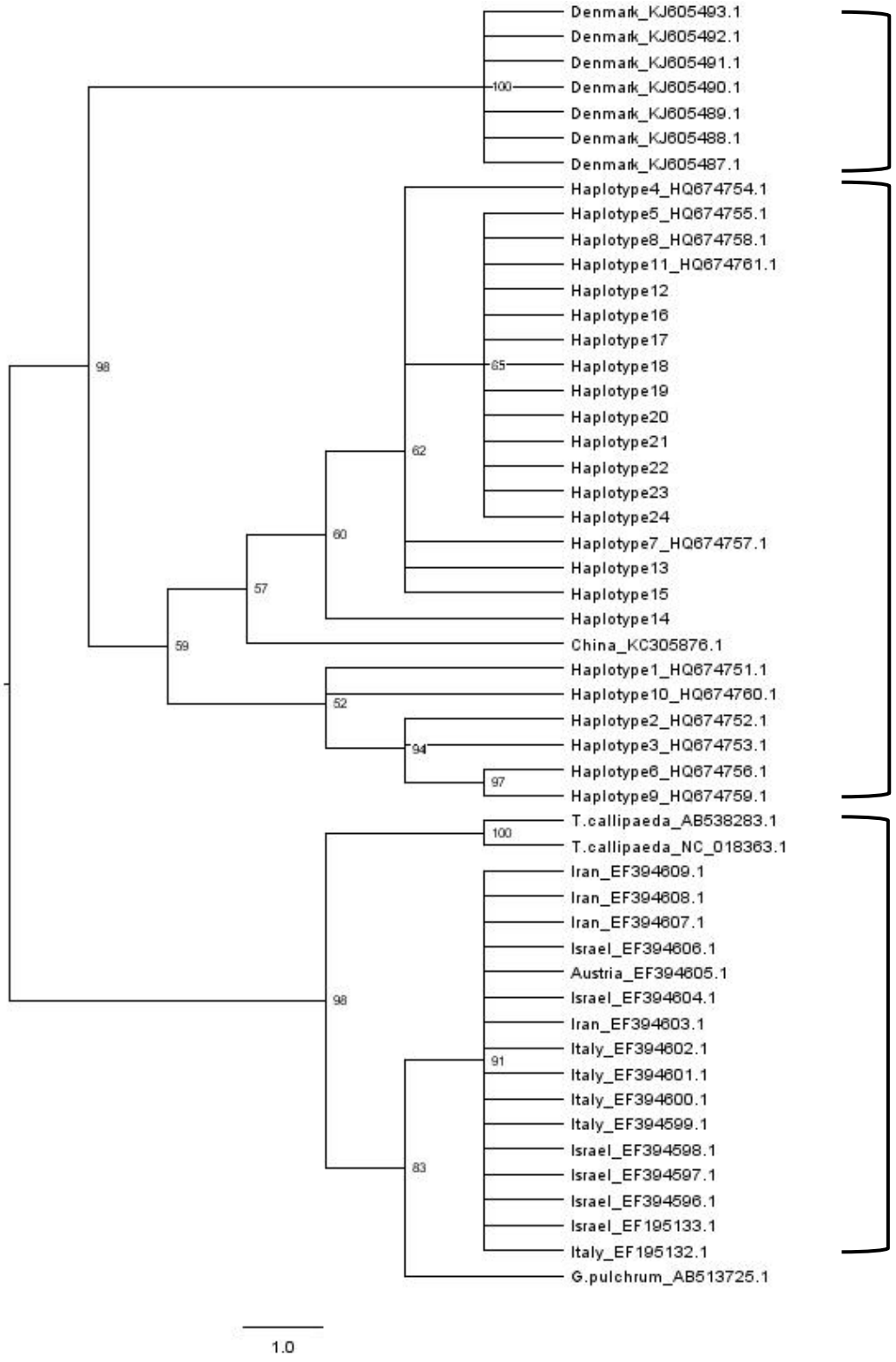


Figure 2.8: Phylogenetic tree of Bayesian inference of *cox1* sequences from *S. lupi* derived from different continents using the MCMC method. Posterior probabilities are shown at the nodes. The scale bar indicates distance. Three groups indicated by A, B and C.

A pairwise differences table was constructed (table 2.2) to compare the differences between the sequences from the various continents. When comparing local South African sequences the pairwise difference is in the range of 0.00 and 0.034. Similar to that found between the two *Thelazia* isolates where the pairwise distance is 0.006.

When compared to sequences of *S. lupi* from domestic dogs from Europe, the South African *S. lupi* sequences range from 0.102 to 0.120. The *S. lupi* sequences from red foxes from Denmark have a pairwise distance range from 0.108 to 0.137 when compared to the South African *S. lupi* sequences and 0.120 to 0.130 when compared to *S. lupi* sequences from European domestic dogs. When comparing all the *S. lupi* samples to the two outgroups the range is between 0.141 and 0.168. In the study from Denmark it was remarked that the nematodes from the red fox may be a cryptic species of *Spirocerca* (AL-SABI *et al.* 2014) since the pairwise difference to European sequences are almost as high as sequences compared to an outgroup. The same may apply to the South African *Spirocerca* nematodes. In addition this data supports the three groupings that can be seen in figures 2.7 and 2.8.

Table 2.2: Pairwise distance in *cox1* of the *S. lupi* haplotypes compared to sequences from the NCBI database of *S. lupi* from other parts of the world as well as two *Thelazia* isolates for comparison. The region from where the samples originate as well as the accession numbers are shown except for the haplotypes from this study.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Haplotype12																				
2	Haplotype13	0.006																			
3	Haplotype14	0.013	0.006																		
4	Haplotype15	0.020	0.013	0.020																	
5	Haplotype16	0.006	0.013	0.020	0.027																
6	Haplotype17	0.013	0.020	0.027	0.034	0.020															
7	Haplotype18	0.006	0.013	0.020	0.027	0.006	0.020														
8	Haplotype19	0.000	0.006	0.013	0.020	0.006	0.013	0.006													
9	Haplotype20	0.000	0.006	0.013	0.020	0.006	0.013	0.006	0.000												
10	Haplotype21	0.006	0.013	0.020	0.027	0.013	0.020	0.013	0.006	0.006											
11	Haplotype22	0.006	0.013	0.020	0.027	0.013	0.020	0.013	0.006	0.006	0.013										
12	Haplotype23	0.013	0.020	0.027	0.034	0.020	0.027	0.020	0.013	0.013	0.020	0.020									
13	Haplotype24	0.013	0.020	0.027	0.034	0.020	0.027	0.020	0.013	0.013	0.020	0.020	0.027								
14	Iran_EF3946091	0.111	0.102	0.102	0.102	0.120	0.111	0.119	0.111	0.111	0.120	0.120	0.129	0.120							
15	Israel_EF3946061	0.111	0.102	0.102	0.102	0.120	0.111	0.119	0.111	0.111	0.120	0.120	0.129	0.120	0.000						
16	Austria_EF3946051	0.111	0.102	0.102	0.102	0.120	0.111	0.119	0.111	0.111	0.120	0.120	0.129	0.120	0.000	0.000					
17	Italy_EF3946021	0.111	0.102	0.102	0.102	0.120	0.111	0.119	0.111	0.111	0.120	0.120	0.129	0.120	0.000	0.000	0.000				
18	China_KC3058761	0.034	0.027	0.020	0.042	0.042	0.049	0.041	0.034	0.034	0.042	0.042	0.049	0.049	0.094	0.094	0.094	0.094			
19	Denmark_KJ6054931	0.108	0.117	0.108	0.137	0.117	0.108	0.116	0.108	0.108	0.117	0.117	0.126	0.127	0.130	0.130	0.130	0.130	0.099		
20	*T.callipaeda* _NC_0183	0.138	0.148	0.148	0.148	0.148	0.158	0.147	0.138	0.138	0.148	0.148	0.157	0.148	0.131	0.131	0.131	0.131	0.168	0.149	
21	*T.callipaeda* _AB53828	0.148	0.158	0.158	0.158	0.158	0.168	0.157	0.148	0.148	0.158	0.158	0.167	0.158	0.141	0.141	0.141	0.141	0.179	0.159	0.006

CONCLUSION

This study shows what appears to be an increase in genetic diversity in *cox1* of *S. lupi* across various regions of South Africa over time. This increase in diversity might be what is causing increased cases of reported spirocercosis. However, increased awareness and vigilance of the parasite cannot be excluded. Greater diversity is problematic as it may lead to greater anthelminthic resistance and better adaptation of the parasite. There appears to be some isolated mutation within each region but there are also indications of gene flow between the four regions studied. Coprophagous beetles may be the cause of this spread but considering the distance between regions, and the number of similar haplotypes, it seems more likely that humans are the main cause of distribution. People can transport their pets to new regions and consequently transport *S. lupi* since symptoms of spirocercosis are not always immediately evident. Furthermore wild carnivores such as the black backed jackal may also increase the transmission of the parasite as there are shared haplotypes between *S. lupi* from these species and the domestic dog. More genetic studies are needed to further understand the dynamics of *S. lupi* anthelminthic resistance and the dynamics of how it is spread across the country.

Comparisons between *Spirocerca cox1* of different continents indicate that there might be a cryptic species of *Spirocerca* in South Africa. Since only a very small segment of the *cox1* was used in this analysis further studies will have to be conducted to clearly delineate the various potential species. It is recommended that either the whole *cox1* region is used for analysis or that other regions of the genome as well as ribosomal DNA is used for clearer and more accurate results. Sequences from wolves might also shed some light on whether there is species variation between the different continents as the first instance of *S. lupi* was recorded from a wolf.

APPENDIX 1

Sample	Source	NCBI Description	Max score	Total score	Query cover	E alue	Identity	Accession	Hap	Date
D30W3	Dog, Durban	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	7/4/2010
J3W2	Jackal, Kimberley	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	unknown
P18W1	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	6/5/2005
P18W3	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	6/5/2005
P19W3	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	30/6/2005
P30W1	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	28/4/2006
P33W1	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	13/7/2005
P7W1	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	2/11/2005
P9W1	Dog, Pretoria	*Spirocerca lupi* haplotype 1 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674751.1	1	15/6/2005
G11W2	Dog, Grahamstown	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	May-12
J2W1	Jackal, Kimberley	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	unknown
P12W3	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	23/3/2005
P15W1	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	17/10/2005
P18W2	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	6/5/2005
P20W3	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	20/4/2006
P21W2	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	21/2/2005
P24W1	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	22/9/2005
P2W3	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	12/9/2012
P4W2	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	28/5/2012
P5W3	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674754.1	4	27/7/2005
J3W3	Jackal, Kimberley	*Spirocerca lupi* haplotype 9 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674759.1	9	unknown
P17W1	Dog, Pretoria	*Spirocerca lupi* haplotype 9 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674759.1	9	23/1/2007
P19W1	Dog, Pretoria	*Spirocerca lupi* haplotype 9 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674759.1	9	30/6/2005
P26W1	Dog, Pretoria	*Spirocerca lupi* haplotype 9 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674759.1	9	2/5/2005
P26W2	Dog, Pretoria	*Spirocerca lupi* haplotype 9 *cox1* gene, partial cds; mitochondrial	640	640	100%	6E-180	100%	HQ674759.1	9	2/5/2005
D1W2	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	17/5/2012
D1W3	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	17/5/2012
D2W2	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	unknown

Sample	Source	NCBI Description	Max score	Total score	Query cover	E value	Identity	Accession	Hap	Date
D30W1	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	7/4/2010
D30W2	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	7/4/2010
G11W3	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	May-12
G2W2	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	18/4/2012
G5W2	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	1/5/2012
G5W3	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	1/5/2012
G6W3	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	1/5/2012
J1W1	Jackal, Kimberley	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	unknown
J3W1	Jackal, Kimberley	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	unknown
P11W2	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	28/2/2007
P15W3	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	17/10/2005
P20W2	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	20/4/2006
P21W3	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	21/2/2005
P23W1	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	4/4/2006
P5W2	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	27/7/2005
P8W2	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674755.1	12	16/8/2005
J2W2	Jackal, Kimberley	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674754.1	13	unknown
J2W3	Jackal, Kimberley	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	634	634	100%	3E-178	99%	HQ674754.1	14	unknown
P16W3	Dog, Pretoria	*Spirocerca lupi* haplotype 4 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674754.1	15	8/12/2006
P26W3	Dog, Pretoria	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	16	2/5/2005
G2W1	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	623	623	100%	6E-175	99%	HQ674755.1	17	18/4/2012
G6W1	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	18	1/5/2012
G8W1	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	19	1/5/2012
G11W1	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	20	May-12
G12W1	Dog, Grahamstown	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	21	May-12
D1W1	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	628	628	100%	1E-176	99%	HQ674755.1	22	17/5/2012
D2W1	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	623	623	100%	6E-175	99%	HQ674755.1	23	unknown
D3W2	Dog, Durban	*Spirocerca lupi* haplotype 5 *cox1* gene, partial cds; mitochondrial	623	623	100%	6E-175	99%	HQ674755.1	24	7/4/2010

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Chapter 3:

Spirocerca lupi egg DNA extraction method and wild carnivore faecal analysis

ABSTRACT

There has been limited research on *Spirocerca lupi* among wild carnivores in South Africa. Studies have shown that black backed jackals (*Canis mesomelas*) are hosts for *S. lupi* as well as some smaller mammals including foxes. Faecal analysis may be a reasonable approach when investigating the incidence of *S. lupi* among wild carnivores. Although this method does have limitations, such as irregular egg shedding, it provides a non-invasive and cost effective means to study this parasite. Of particular interest is the endangered wild dog which may also be a host to *S. lupi* but have never been studied to determine if they harbour this particular parasite. In this study, *S. lupi* eggs were successfully isolated from dog faecal samples but the DNA could not be isolated from the eggs. This was attributed to either the low DNA concentrations obtained or that the eggs were not disrupted sufficiently. A nested PCR approach may be the answer to successfully isolating DNA from *S. lupi* eggs as this will allow for the amplification of very low levels of DNA.

INTRODUCTION

There is very little information available on the incidence of *Spirocerca lupi* among wild carnivores in South Africa. In Africa, *S. lupi* has been observed in the black backed jackal (*Canis mesomelas*). Five jackals were examined where prominent aortic lesions were present in four of the jackals and oesophageal masses were present in all five of the jackals (BRODEY *et al.* 1977). In the same study three spotted hyenas (*Crocuta crocuta*) were also examined but they did not show any signs of *S. lupi* infection. A possible explanation for the infection in the jackals was attributed to coprophagy that was observed among the jackals as well as some of the local dogs (BRODEY *et al.* 1977). Jackals are also known to consume wild birds and chickens as well as insects (DORST and DANDELOT 1970; KIRKOVA *et al.* 2011), which are all potential hosts of *S. lupi* (BRODEY *et al.* 1977). Contrary to these findings, a more recent study showed that spotted hyena may potentially be hosts for *S. lupi*. Eggs were found in the faeces of spotted hyena that resemble those of *S. lupi* with an approximate size of 36 x 12 µm (ENGH *et al.* 2003). In a different study a cheetah cub was also observed with *S. lupi* larvae tracking along the aorta (MURRAY *et al.* 1964).

A study in Sredna Gora, Bulgaria revealed that there was no infection with *S. lupi* among a group of 78 golden jackals (*Canis aureus*) (KIRKOVA *et al.* 2011). This lack of infection was attributed to the feeding habits of the jackals. The jackals were found to feed mostly on carrion (79.5%). In this same study 167 foxes (*Vulpes vulpes*) and 40 wild cats (*Felis silvestris*) were also examined. Among these there was a 24.6% incidence of *S. lupi* among the foxes and a 27.3% incidence of *S. lupi* among the wild cats. It was found that these animals consumed mainly rodents (foxes, 50% and wild cats 82.5%). Curiously the wild cats did not seem to consume any insects although they had a high rate of infection. Foxes were found to consume insects (0.8%) whereas jackals also did not consume any insects (KIRKOVA *et al.* 2011). A recent study in Denmark discovered a unique, genetically distinct *Spirocerca* nematode in a red fox (*Vulpes vulpes*) (AL-SABI *et al.* 2014). This was the first reported incidence of a red fox with *Spirocerca* infection in Denmark. The nodules of spirocercosis in foxes seems to mainly be situated in the gastric region as opposed to the oesopahgus as is most common in the domestic dog (FERRANTELLI *et al.* 2009; DIAKOU *et al.* 2012). Table 3.1 shows some of the African mammals that are known to be hosts of *S. lupi* (ROUND 1968).

Table 3.1: Other known *S. lupi* infections among African wild mammals

Life stage	Mammal species	Common name
Adult worm	Canis sp. *Felis serval* *Felis libyca* *Fennecus zerda* *Vulpes vulpes*	jackal Serval African wild cat fennec fox or fennec red fox
Larva	*Mustela nivalis* *Poecilictus libyca*	least weasel striped weasel

Collecting DNA from nematode eggs in faeces is a non-invasive method of population screening which should cause minimum disturbance of the animals wellbeing and ecology (NONAKA *et al.* 2011). On the other hand faecal examination may also have some limitations and is therefore best used in combination with molecular techniques. A combination approach can provide more accurate results and also provide researchers with a tool for surveying zoonotic and other parasitic infections in dogs, as has been shown previously (DAVIDSON *et al.* 2009; NONAKA *et al.* 2011). These methods should also be applicable to wild carnivores in South Africa.

Some of the limitations of faecal analysis include sporadic egg shedding as a study in Kenya showed. In this study, faecal examination of 145 native dogs whose oesophageal nodules had open apertures revealed that only 56% had *S. lupi* eggs in their faeces (BRODEY *et al.* 1977). Unfortunately collecting adult nematodes requires risky and costly surgical procedures. Usually adult nematodes are obtained through post mortem examinations, which prevents screening of live animals for *S. lupi* infections. This is particularly problematic when wild carnivore species are involved. Considering this, nematode DNA from eggs in faeces is therefore currently still the most feasible, non-invasive and economical method for collecting nematode DNA from wild carnivores despite the limitations where false negatives may be recorded.

Various methods have been devised to isolate DNA from nematode eggs in faeces. These methods all utilize different egg disruption techniques which may be chemical or physical. The chemical disruption techniques usually employ high temperatures and a lysing buffer to assist with egg disruption. Some examples of this are from the isolation of DNA from *Parelaphostrongylus odocoilei*. The methods employed here include an extraction buffer solution with a single egg that is heated to 90°C for 15 minutes (BRYAN *et al.* 2010). Another example is the disruption of *Schistosoma mansoni* eggs with a modified version of the ROSE method (STEINER *et al.* 1995). This method includes the slightly modified ROSE buffer (10 mM Tris-HCl, pH 8.0; 312.5 mM EDTA, pH 8.0; 1% sodium lauryl sarkosyl; and 1% polyvinylpolypyrrolidone (PVPP, water insoluble)) that is added to the faecal supernatant and incubated at 95°C for 20 min (PONTES *et al.* 2002). For physical disruption a bead homogenizer may be used. For example in the isolation of *Ostertagia ostertagi* eggs from cattle faeces different lysing matrices (6.5 mm ceramic beads with 0.25 g ceramic chips, 6mm glass beads with 0.35 g glass powder and 0.90 g zirconia/silica beads only) were tested for their disruption capability. It was found that the ceramic beads were most effective in disrupting the *Ostertagia ostertagi* eggs (HARMON *et al.* 2006).

Faecal flotation is commonly used as a diagnostic tool for spirocercosis as well as other nematode infections. A highly concentrated salt solution is added to a faecal sample and centrifuged at a defined relative centrifugal force for a certain period of time. Through this the eggs of the nematodes are separated from the faecal sample and suspended in the supernatant. The supernatant is examined microscopically and the eggs identified. Some DNA extraction protocols include this faecal flotation method in order to isolate eggs from the faecal material. For example, for the extraction of DNA from *Taeniid* eggs, the eggs were detected with faecal flotation (262 mg/ml $ZnCl_2$ and 275 mg/ml NaCl) and were washed from the slides with 0.9% NaCl and frozen at -80 °C for further analysis (DYACHENKO *et al.* 2008). For *S. lupi*, a modified faecal flotation method has already been described which allows for the optimum number of eggs to be isolated (CHRISTIE *et al.* 2011).

A PCR based detection method for *Echinococcus multilocularis* eggs also uses a concentrated salt solution to remove the eggs from the rest of the faecal material (MATHIS *et al.* 1996). Briefly, fox faeces were passed through a 100 µm sieve then re-suspended in a zinc chloride solution (1.45 g/ml) and centrifuged. The supernatant was passed through a series of sequential sieves until the eggs were caught on the final 20 µm sieve and washed into a tube for further analysis (MATHIS *et al.* 1996).

Veterinarians in and around the Tshwane Metropolitan area are of the opinion that *S. lupi* is more prevalent in the areas surrounding the National Zoological Gardens (NZG) in Pretoria (personal communication). The wild carnivores of the NZG may serve as reservoirs of *S. lupi* thus affecting the prevalence of *S. lupi* in the adjacent areas. Wild animals may increase the number of definitive and intermediate host species and they may also increase the parasites range (MACPHERSON 1994). The aim of this study was to develop a non-invasive faecal method to screen wild carnivores for *S. lupi* infection while extracting DNA for future genetic analysis. Animals that have been considered for this study include the striped hyena (*Hyaena hyaena*), spotted hyena (*Crocuta crocuta*), cheetah (*Acinonyx jubatus*), lion (*Panthera leo*) and the African wild dog (*Lycaon pictus*). The strategy was to develop a suitable method to extract nematode DNA from eggs in faeces. This study therefore focuses on DNA extraction methods, where several methods were tested and modified in order to determine if it would be possible to extract DNA from *S. lupi* eggs in faeces.

MATERIALS AND METHODS

Faecal samples

Dog faecal samples were obtained with consent from pet owners in Pretoria (Tshwane Metropole) whose dog(s) had been diagnosed with *S. lupi*. Samples were usually collected early in the morning and stored at 4°C. Wild carnivore faecal samples were obtained from the National Zoological Gardens (NZG) in Pretoria (Tshwane Metropole). Samples were collected by personnel of the NZG and placed in plastic containers for collection on the same day. Fresh faecal samples were kept at 4°C until DNA extraction could be performed. Samples were incubated for a maximum of 72 hours before DNA extraction. Jackal faecal samples were obtained from the Kruger National Park. They were stored in 70% ethanol at 4°C.

Nematode samples

S. lupi adult worms were obtained from post mortem examination of dogs presenting with or suspected of having spirocercosis at the Onderstepoort Veterinary Academic hospital between 2005 and 2007. These nematodes were stored in 70% ethanol at 4°C.

Adult nematode DNA extraction

Spirocerca lupi nematodes that were obtained between 2005 and 2007 were used as positive controls in all PCR reactions. Before DNA extraction, the nematodes were washed three times in 96% ethanol in order to remove any residual blood or tissue. Once washed, the nematodes were crushed in liquid nitrogen after which DNA extraction was performed on each nematode using the DNeasy® blood and tissue kit (Qiagen). The protocol for the purification of total DNA from animal tissue was followed according to the manufacturers instructions.

Faecal DNA extraction

Faecal DNA extraction was performed using the QIAmp® DNA stool mini kit (Qiagen) according to the manufacturers instructions for the protocol for pathogen detection.

Faecal flotation

The faecal flotation method used for *S. lupi* was previously described by Christie et al. (2011). Two grams of faeces were homogenised in a 5 ml solution of 1.22 g/ml sodium nitrate with a spatula. The solution was then centrifuged for 10 min at 1400 g. A 100 µl sample of the supernatant was examined with an optical microscope at 100x magnification to screen for the presence of nematode eggs (CHRISTIE *et al.* 2011).

Bead homogenization

Bead homogenization was performed with Lysing Matrix E (MP Biomedicals). The beads and samples were mixed in the provided 2 ml tube by inverting the tube a few times or briefly vortexing. The tubes were homogenized in a FastPrep 24 tissue and cell homogenizer (MP Biomedicals) for 20 sec at speed setting 4.5 (see optimization results).

DNA precipitation

DNA precipitation was performed by taking 50 µl of the final DNA elution (after faecal DNA extraction) with 300 µl absolute ethanol and 3 µl 3 M sodium acetate at pH 4.6. The solution was incubated at -20°C for one hour or overnight to allow for the DNA to precipitate. The solution was centrifuged for 30 min at 13000 rpm using a bench top centrifuge (SimpliAmp™ Thermal Cycler, Applied Biosystems™). The supernatant was removed and the pellet was washed twice in 250 µl 70% ethanol. Between each washing step the solution was centrifuged for 5 min at 13000 rpm. The pellet was dried at 80°C for 3 min or overnight at 4°C and re-suspended in 5 µl ultra-high quality water. The entire 5 µl was used as template in the polymerase chain reaction regardless of DNA concentration.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using previously described universal nematode primers. These primers, namely, JB3 (5'- TTTTTTGGGCATCCTGAGGTTTAT) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG) amplify the Cytochrome C oxidase 1 region (BOWLES *et al.* 1992) and have previously been shown to amplify *S. lupi cox1* DNA, yielding an amplicon of approximately 440 bp (DE WAAL *et al.* 2012). The PCR reaction mixture consisted of 2 mM $MgCl_2$, 0.2 mM of each dNTP, 100 pmol of each primer, 1 unit of GoTaq® DNA polymerase (Promega) and 1x green Flexi buffer supplied with the polymerase. Faecal sample DNA was assessed at a high concentration of 100-300 ng. The final reaction mixture was made up to 50 µl with ultra-high quality water. For a positive control approximately 100 ng of *S. lupi* adult nematode DNA was used. The reaction cycle consisted of an initial 5 min denaturation step at 95°C followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing

at 58°C and 1 min elongation at 72°C. A final elongation of 5 min was performed at 72°C to facilitate full extension of the amplicons.

Semi nested PCR

A semi nested PCR was performed using primers COIintF (5' - TGATTGGTGGTTTTGGTAA) (CASIRAGHI *et al.* 2001) and JB4.5. The same reaction conditions were used as previously described, only the annealing temperature was lowered to 52°C. This reaction was followed by a PCR reaction with primers JB3 and JB4.5 as previously described.

Agarose gel electrophoresis analysis

PCR reactions were analysed by gel electrophoresis using an agarose gel made from 1% agarose, 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and 0.4 µg/ml ethidium bromide. All analyses were performed at 100V for approximately 40 min to ensure adequate separation. Gels were examined under 70% fluorescent light and amplicons were excised from the agarose gel. These were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturers instructions.

NanoDrop spectrophotometry

Nucleic acid purity was assessed by determining the ratio of absorbance at 260/280 nm using a NanoDrop® ND1000 spectrophotometer (Thermo Scientific) according to the manufacturers instructions.

Cloning

Cloning was performed using the StrataClone PCR cloning kit (Agilent technologies) with the StrataClone SoloPack competent cells (Agilent technologies) according to the manufacturers

instructions. Ligation reactions were incubated at 4°C overnight to ensure adequate ligation before transformation was performed. Cells were plated on Luria-Bertani medium agar plates supplemented with ampicillin, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside). Blue-white screening was used for selection of recombinants.

Sequencing

Quarter sequencing reactions consisted of Big Dye terminator v3.1, 1x Big Dye dilution buffer and 3.2 pmol of the commercially available pUCM13F primer (Promega). Approximately 200-250 ng of plasmid DNA was used in the sequencing reaction. The final volume was made up to 10 µl with Ultra High quality water. The sequencing reaction started with an initial 1 min denaturation step at 96˚C followed by 25 cycles of 95˚C for 10 sec, 50˚C for 20 sec and 60˚C for 4 min. The sequences were purified after the cycle reaction by placing the entire 10 µl reaction in 50 µl absolute ethanol with 3 µl 3 M sodium acetate at pH4.6. The samples were incubated overnight in -20˚C to allow the DNA to precipitate. Following precipitation, the samples were centrifuged for 30 min at 13000 rpm and the pellet washed twice in 250 µl 70% ethanol. The samples were centrifuged for 5 min at 13000 rpm between each wash step to ensure the pellet was not re-suspended. The pellet was dried overnight at 4˚C and processed at the ACGT African Centre for Gene Technologies DNA sequencing facility at the University Of Pretoria using the ABI 3100 and ABI 3500 systems.

Data analysis

The CLC bio software (CLC bio) along with the program BioEdit (HALL 1999) were used to edit sequences and determine sequence quality. Sequences were analysed through the Basic Local Alignment Search Tool (BLAST) (ALTSCHUL *et al.* 1990) hosted by the *National Centre for Biotechnology Information* (NCBI). Sequences were converted to FASTA format before a standard nucleotide BLAST with database selected as 'other'. The BLAST search was optimized for highly similar sequences (megablast).

FAECAL SAMPLE PREPARATION AND DNA EXTRACTION METHODS

Method 1: Faecal flotation (FF)

The Faecal flotation method used for *S. lupi* was previously described by Christie et al. (2011). Briefly two grams of faeces were homogenised in a 5 ml solution of 1.22 g/ml sodium nitrate with a spatula. The solution was then centrifuged for 10 min at 1400 g. A 100 µl sample of the supernatant was examined with an optical microscope at 100x magnification to screen for the presence of nematode eggs (CHRISTIE *et al.* 2011). DNA extraction was performed using the QIAmp® DNA stool mini kit (Qiagen) according to the manufacturers instructions for the protocol for pathogen detection. The protocol was adapted and only 1 ml (instead of 1.4 ml) buffer ASL (QIAmp DNA stool mini kit, Qiagen) was used to accommodate 500 µl of the flotation supernatant if eggs were present. Two separate successive 100 µl elutions were made instead of the recommended single 200 µl elution to allow for higher DNA concentrations.

Method 2: Faecal flotation and bead homogenization (FFH)

This method uses faecal flotation as explained in method 1 but instead the 500 µl flotation supernatant was placed in a 2 ml screw cap tube containing Lysing Matrix E (MP Biomedicals). The beads and supernatant were mixed by inverting the tube a few times or briefly vortexing. The tubes were homogenized in a FastPrep 24 tissue and cell homogenizer (MP Biomedicals) for 20 sec at speed setting 4.5 (see optimization results). Once the sample was homogenized, 1 ml buffer ASL was added to the 2 ml tube before DNA extraction was performed with the QIAmp DNA stool mini kit.

Method 3: Sequential sieving (SeqS)

The method proposed by Mathis *et al.* (1996) used a sieving protocol to remove *Echinococcus multilocularis* eggs from fox faeces (MATHIS *et al.* 1996). This method was modified slightly for this study. Briefly, approximately 20 ml of faeces was placed in a plastic centrifugation tube and distilled

water was added to a final volume of 50 ml. The samples were left at room temperature for 30 min to allow the faeces to soften. The samples were then thoroughly homogenized through vigorous vortexing. The homogenized sample was passed through a tea strainer followed by sequential sieving through sieves of descending pore sizes (200 µm followed by a 100 µm sieve). The sieves were placed between two polystyrene cups of which the bottoms were removed. The filtrate was centrifuged at 1000 g for 10 min. The pellet was re-suspended in 5 ml of sodium nitrate solution of a density of 1.22 g/ml and centrifuged for 10 min at 1400 g (faecal flotation method). The supernatant was examined under a light microscope to determine if *S. lupi* eggs were present. The supernatant was filtered through 50 µm followed by a 10 µm sieve. *S. lupi* eggs have an approximate size of 35 X 15 µm (VAN DER MERWE *et al.* 2008). The fraction contained on the 10 µm sieve would be expected to contain the eggs was washed off with 1 ml buffer ASL (QIAmp DNA stool mini kit, Qiagen) and collected in a 2 ml eppendorf tube. DNA extraction was performed on the eggs in the tube as previously described using the QIAmp DNA stool mini kit.

Method 4: Sequential sieving and bead homogenization (SeqSH)

This method employs a similar sieving protocol as described for method 3, but instead of washing the sieve into an empty 2ml eppendorf tube for direct DNA extraction the sieve was washed off into a 2 ml tube containing Lysing Matrix E (MP Biomedicals). The beads and eggs were mixed by inverting the tube several times or briefly vortexing. The tubes were then placed in a FastPrep 24 tissue and cell homogenizer (MP Biomedicals) for 20 sec at speed 4.5. Homogenization conditions were selected based on optimization results from method 2. DNA extraction was performed on the homogenized sample. The sieves and tea strainers were cleaned after use by soaking in 2-3 % sodium hypochlorite overnight followed by drying at 60˚C for 6-24 hours.

Method 5: Simplified sieving and bead homogenization (SSH)

This method is an adaptation of method 3. Approximately 20 ml of faeces was mixed with distilled water to a final volume of 50 ml. The suspension was then mixed by vortexing or homogenized with a

spoon or spatula. The faecal sample was sieved through a single layer of gauze and through a 200 µm sieve. Approximately 15 ml of water was used to wash out the beaker. Once the sample had passed through the gauze and the 200 µm sieve, the filtrate was centrifuged for 10 min at 2000 g and the supernatant discarded. The pellet was re-suspended in 5 ml $NaNO_3$ (1.22 g/ml) and centrifuged for 10 min at 1400 g. The supernatant was removed and placed in a new centrifugation tube. Approximately 100 µl of the supernatant was examined under a light microscope at 100X to 400X magnification to determine if any eggs were present. Water was added to the supernatant to a final volume of 20-25 ml in order to dilute the salt solution. The solution was centrifuged at 2000 g for 10 min. The final pellet was re-suspended in 1 ml buffer ASL and transferred to a tube with Lysing Matrix E (MP Biomedicals). The beads and re-suspended pellet were mixed by inverting the tube a few times or vortexing for a few seconds. The tubes were placed in a FastPrep 24 tissue and cell homogenizer (MP Biomedicals) for 5 sec at speed setting 4 (based on results from method 4). DNA extraction was performed on the homogenized sample as described previously with the QIAmp DNA stool mini kit.

Method 6: Simplified sieving (SS)

This method used the same sieving technique as method 5 but without the bead homogenization step. In other words, once the final pellet was re-suspended in 1 ml buffer ASL it was transferred to a normal 2 ml Eppendorf tube instead of the Lysing Matrix E tube, and DNA extraction was performed directly on the final pellet with the QIAmp DNA stool kit (Qiagen).

RESULTS AND DISCUSSION

Adult *S. lupi* nematode DNA extractions

DNA was isolated from adult *S. lupi* nematodes obtained after necropsy at the Onderstepoort Veterinary Academic Hospital. Adult *S. lupi* DNA was extracted to provide DNA for positive controls in PCR reactions. DNA extractions were performed with a commercial kit in order to ensure high DNA quality. The DNA concentrations obtained after DNA extraction were determined using the NanoDrop® ND1000 spectrophotometer (table 3.2). DNA concentration obtained ranged between 76.9 and 201.0 ng/µl. The absorbance ratios at 260/280 nm were in the order of 1.8 indicating relatively pure DNA.

Table 3.2: Final DNA concentration obtained for the adult *S. lupi* nematodes

Sample	ng/µl	260/280
183-07	88.3	1.82
1108-06	166.7	1.76
11061	121.4	1.79
20-06	76.9	1.85
822-05	85.7	1.81
541-05	95.0	1.79
9133	111.9	1.79
343-06	101.9	1.73
105-05	92.0	1.78
410-06	201.0	1.71

Faecal DNA extractions

The aim of the research presented in this chapter was to develop a standardized, repeatable method that can be used to safely screen dogs as well as other carnivores for *S. lupi* infection through faecal DNA extraction and PCR. The main strategy was to isolate the eggs from the faecal samples then extract

DNA from the eggs directly. This section describes the results obtained for the various methods tested and how each method was modified as was deemed necessary. A summary of the various methods used in this study is shown in table 3.3.

Table 3.3: Summary of the various DNA extraction methods used

	Faecal flotation (FF)	**Bead homogenization (H)**	**Sequential Sieving (SeqS)**	**Simplified sieving (SS)**
Method 1: Faecal flotation (FF)	✓			
Method 2: Faecal flotation and bead homogenization (FFH)	✓	✓		
Method 3: Sequential sieving (SeqS)	✓		✓	
Method 4: Sequential sieving and bead homogenization (SeqSH)	✓	✓	✓	
Method 5: Simplified sieving and bead homogenization (SSH)	✓	✓		✓
Method 6: Simplified sieving only (SS)	✓			✓

Method 1: Faecal flotation (FF)

Faecal flotation was used in order to isolate the eggs from the faecal samples before DNA extraction. Since very few eggs were present in the faecal sample, using the flotation method ensures that as many eggs as possible are removed. Figure 3.1 shows an egg at 1000x magnification after faecal flotaion.

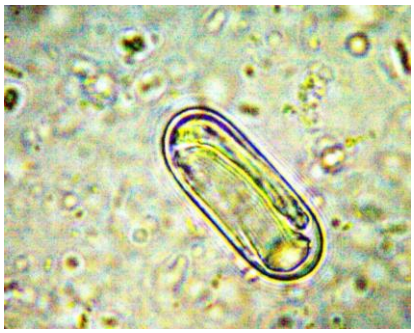


Figure 3.1: Image at 1000x magnification of an *S. lupi* egg after faecal flotation with the modified centrifugal faecal flotation method. The L1 stage larvae can be seen as it is curled up within the egg. Image courtesy of Janishtha Mitha.

DNA concentrations were not representative of nematode DNA alone but also canine, bacterial and a number of other sources of DNA from various species present in the faecal sample. A commercial kit was used to extract DNA in order to ensure that high quality DNA was obtained. The QIAamp DNA Stool Mini Kit was specifically selected because it contains InhibitEX tablets. These tablets consist of a unique reagent that absorbs compounds that can degrade DNA and inhibit downstream enzymatic reactions. The QIAmp DNA stool mini kit (Qiagen) protocol for pathogen detection was altered slightly in order to use more flotation solution with the intent of increasing the number of eggs in the sample. The initial sample of flotation solution was increased to 500 µl instead of 200 µl and the ASL buffer was decreased to 1 ml instead of 1.4 ml in order to allow the solution to fill the 2ml Eppendorf tube without overflowing. Two successive 100 µl elutions were done instead of the recommended single 200 µl elution in order to obtain higher concentrations of DNA.

The eggs are generally not homogenously distributed within the faecal sample and therefore the small quantities of faeces required by the QIAmp kit, even after volume alterations, seems to be too little to capture sufficient eggs or the eggs were not adequately disrupted for successful DNA extraction. The PCR results for the faecal flotation are shown in figure 3.2. The only amplification obtained was for the positive adult *S. lupi* control (Lane 17).

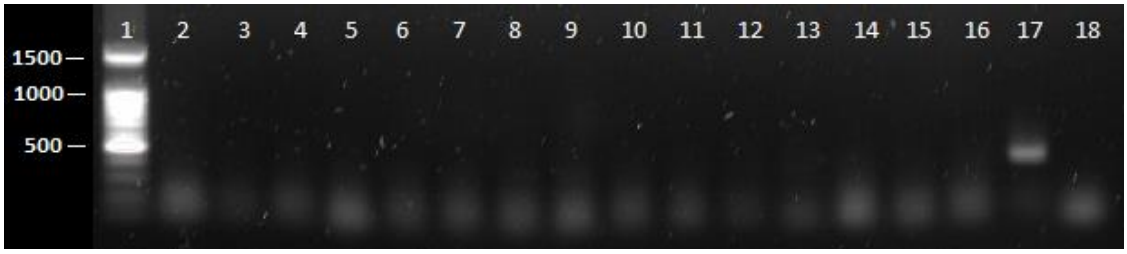


Figure 3.2: Agarose gel of feacal flotation (FF) results. Lane 1, 100 bp ladder; lane 2-16 different faecal samples from dogs; lane 17, positive; lane 18, negative.

Method 2: Faecal flotation and bead homogenization (FFH)

In the second method, bead homogenization was added to the method in order to assist in disrupting the eggs so that DNA could be extracted more adequately, since the faecal flotation method alone was

insufficient. The Lysing Matrix E (MP Biomedicals) was used since this was the recommended bead matrix for homogenizing faecal samples.

Optimization

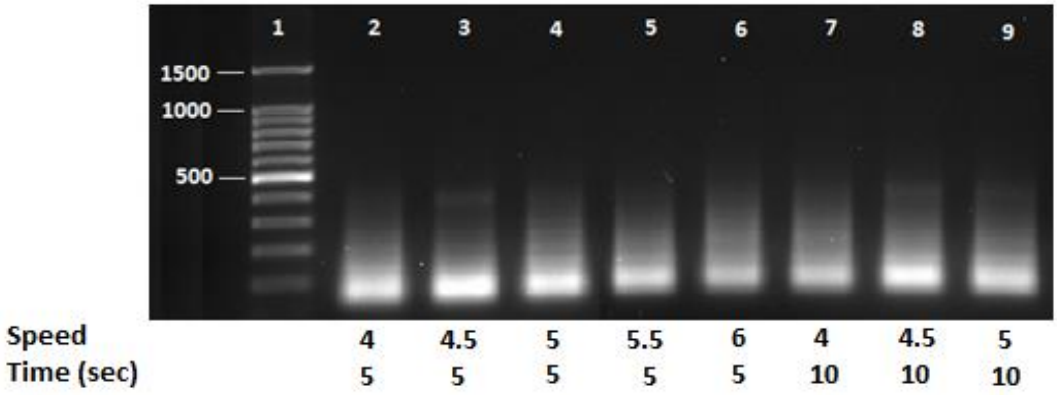
Bead homogenization optimization was performed by using different speed and time settings. A faecal sample from a dog containing *S. lupi* eggs was divided into 15 pieces of 2 g each. Time intervals of 5, 10 and 20 seconds were used with speed settings of 4, 4.5, 5, 5.5 and 6. The results from the nanodrop analysis are shown in table 3.4. The DNA concentrations are representative of total faecal DNA and not just nematode DNA since the faeces contains various bacteria, cells from the carnivore, pollen and potentially many other DNA sources. These DNA concentrations are therefore only an indication of total DNA extracted. Further optimization may still be required as the eggs were not uniformly distributed within the sample and actual egg count was unknown.

Figure 3.3 A and B show the PCR results obtained with the extracted samples. The highest DNA concentration with visible amplification was obtained by the sample that was homogenized for 20 seconds at a 4.5 speed setting (figure 3.3 B, arrow and highlighted in table 3.4).

Table 3.4: DNA yield obtained with various bead homogenization conditions.

Time	**5 seconds**			**10 seconds**			**20 seconds**		
Speed Setting	**260/280**	**260/230**	**ng/µl**	**260/280**	**260/230**	**ng/µl**	**260/280**	**260/230**	**ng/µl**
4	2.13	1.02	30.7	1.89	0.49	29.0	2.02	1.43	35.7
4.5	2.16	1.18	42.3	2.06	0.56	26.9	2.03	0.76	51.9
5	2.09	0.51	38.2	2.18	1.72	33.1	2.08	0.96	51.9
5.5	2.13	1.32	30.4	2.14	0.76	18.5	2.22	1.15	60.7
6	2.02	0.40	29.0	1.95	0.22	40.8	1.96	0.94	56.0

A



B

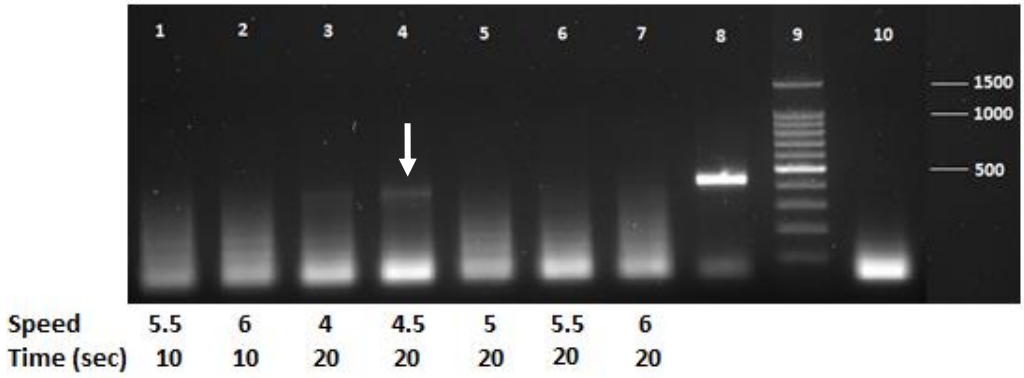


Figure 3.3: A: Agarose gel electrophoresis analysis of PCR for bead homogenization optimization. Lane 1, 100bp ladder. **B:** Agarose gel electrophoresis analysis of PCR for bead homogenization optimization. Lane 8, positive; lane 9, 100 bp ladder; lane 10 negative.

A second PCR was performed with only the samples that were homogenized for 20 seconds to determine if higher amplification could be achieved as the amplicon was very faint on the gel. No amplification was visible in the second PCR as can be seen in figure 3.4. This could be an indication of DNA degradation after DNA extraction since the samples still contained large quantities of protein that may degrade/inhibit the PCR reaction. It was also speculated that the salt solution used in faecal

flotation may be interfering with the DNA extraction procedure, perhaps reacting negatively with one or more of the reagents in the QIAmp DNA stool mini kit.

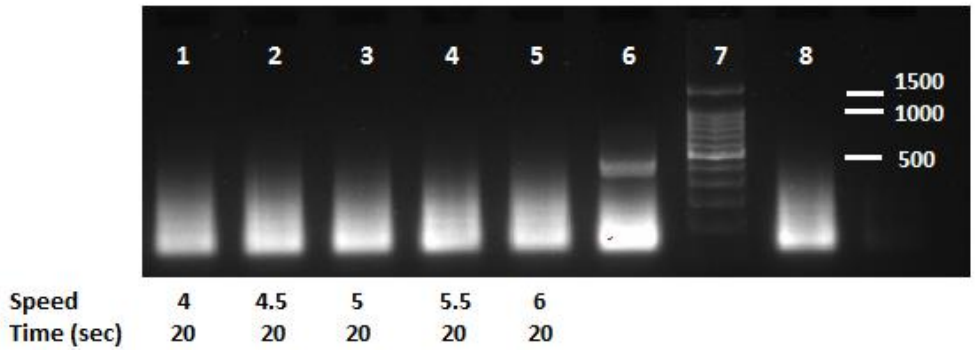


Figure 3.4: Agarose gel electrophoresis analysis of repeat PCR for bead homogenization experiment. Lane 6, positive; lane 7, 100 bp ladder; lane 8, negative control.

Method 3 and 4: Sequential sieving (SeqS) and sequential sieving with bead homogenization (SeqSH)

A dog faecal sample with *S. lupi* eggs was used to test this method. Two samples were prepared, one only with the sequential sieving protocol and one with sequential sieving and bead homogenization. Since faecal flotation was inadequate in removing eggs from the faeces, this sequential sieving method was considered.

The method uses various sized sieves through which a larger quantity of faeces can be sieved. During the sieving process it was found that the eggs could not be captured on the 10 µm sieve because not even water would pass through the 10 µm aperture. This was problematic as the remaining filtered solution volume was too large for the 2 ml bead tubes that were used to homogenize the solution prior to DNA extraction. Therefore only a small amount of the remaining filtered solution was used in DNA extraction and there was no amplification after PCR. In order to increase the DNA yield, DNA precipitation was performed and the pellet was re-suspended in ultra-high quality water for both the bead homogenized sample and the sample that was only sieved. The PCR results after precipitation are shown in figure 3.5. The sample that was only sieved without bead homogenization showed positive amplification. The homogenized sample did not yield any amplification. This may be because the homogenization degraded the DNA. It therefore, appears that a shorter homogenization time at a

slower speed might serve better to break the eggs open without degrading the DNA. However, there seems to be a fine line between optimal egg disruption through bead homogenization and excessive disruption which might lead to DNA degradation.

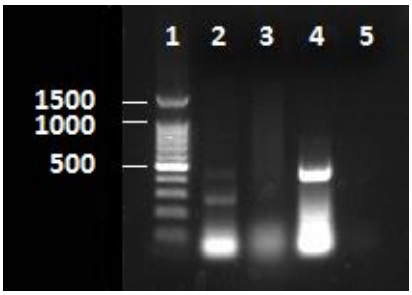


Figure 3.5: Agarose gel electrophoresis for sequential sieving and sequential sieving with bead homogenization after PCR was performed with precipitated DNA. Lane 1, 100 bp ladder; lane 2, SeqS; lane 3, SeqSH; lane 4, positive control; lane 5, negative control.

The amplicons obtained for the non-homogenized sample were excised from the agarose gel and purified for cloning. The transformed clones were selected through blue white screening and mini DNA preparations of the plasmids were prepared. These plasmids were sequenced to determine if the inserts they contain were *cox1* from *S. lupi*. The sequences obtained were analyzed using the NCBI Basic Local Alignment Search Tool (BLAST) and it was revealed that the DNA was from *Gongylonema pulchrum*. Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained. *G. pulchrum* eggs have been observed in a dog in Mami market area in Nigeria (MAHMUDA *et al.* 2012). In addition there have been recently reported cases of *G. pulchrum* in dogs in Brazil (DANTAS-TORRES and OTRANTO 2014). This could be due to a natural infections if the dog consumed one or more of the intermediate hosts which include coprophagous dung beetles and cockroaches (ANDERSON 2000; SATO *et al.* 2005).

The method appears to work well for *G. pulchrum* isolation. *G. pulchrum* eggs are larger, approximately 57 to 62 by 31 to 38 µm (LICHTENFELS 1971), compared to *S. lupi* eggs which are approximately 36 by 12 µm (ENGH *et al.* 2003). The *Gongylonema* eggs may be less resilient than *S. lupi* eggs and were successfully disrupted by bead homogenization. Infective embryonate eggs of *Spirocerca* spp. have been found in topsoil from public squares from two cities within the Argentine Patagonia. Samples

were collected during spring, summer and autumn months with temperatures ranging from 5.2 – 20.4 °C (THEVENET *et al.* 2004). This indicates that *S. lupi* eggs are very resilient since it seems they can survive in soil under the stated conditions for extended periods of time.

Method 5 and 6: Simplified sieving with bead homogenization (SSH) and simplified sieving (SS)

These methods are adaptations of methods 3 and 4 as there were some difficulties with some of the sieves (10 µm and 50 µm) used in the sequential sieving methods. This simplified sieving method also makes use of the faecal flotation method but the flotation solution was removed through washing with water. The purpose being to remove the salt solution as there may have been some interference of the flotation salt with the DNA extraction kit. The method was tested with bead homogenization as well as without the bead homogenization as it was not yet clear what effect the grinding of the beads may have on the DNA. Faecal flotation (method 1 and 2) was also tested along with the simplified sieving method on wild carnivore faecal samples obtained from the NZG. DNA precipitation was performed for all four methods to increase the DNA concentration and ensure that as much sample was used as possible.

It should be noted that the faecal samples received were from different animals of the same species placed in a single container. Different DNA extractions were performed from each container to ensure that as much of the samples were utilized as possible. Therefore different PCR's do not represent different animals but rather samples taken from a single container.

Figure 3.6 shows results from striped hyena faecal samples. Between the four methods tested, namely FF, FFH, (method 1 and 2) SS and SSH (method 5 and 6), the method with the simplified sieving and bead homogenization step produced the best result (lane 11 figure 3.6). These bands were excised, cloned and sequenced. Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained.

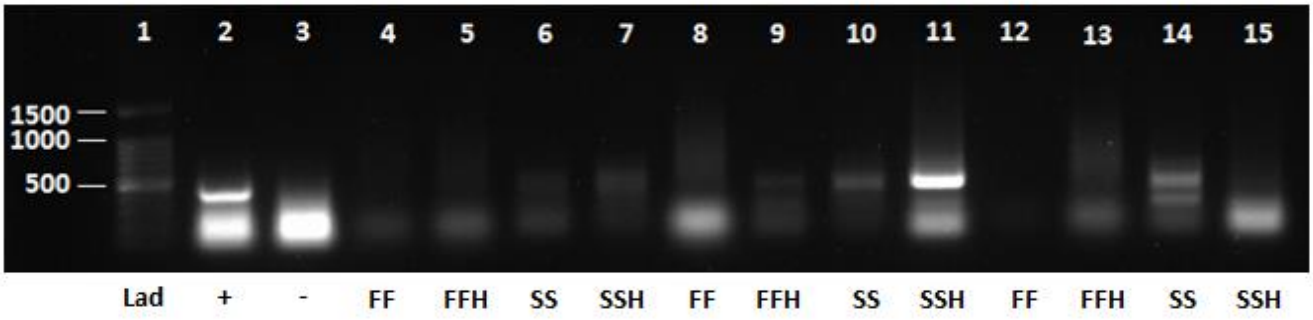


Figure 3.6: Agarose gel analysis to compare the PCR results from various DNA preparation methods using striped hyena faeces.

Figure 3.7 shows the PCR results from cheetah faecal samples. The DNA was extracted using only methods 5 and 6 (SS and SSH). Positive amplification could be seen for the SS method (fig 3.7 lane 5). This amlicon was excised and cloned for sequencing. Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained.

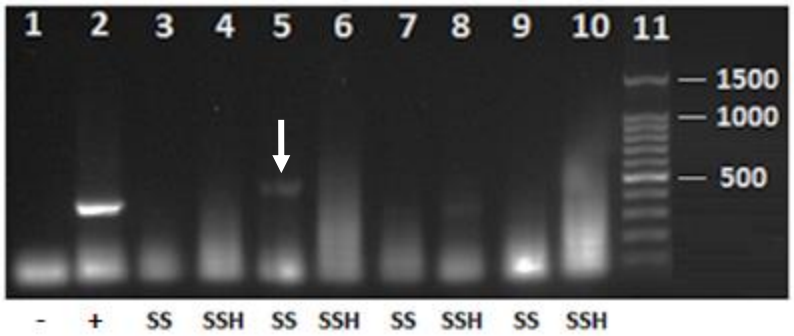


Figure 3.7: Agarose gel showing the PCR of prepared cheetah samples.

Figure 3.8 shows the results for cheetah, spotted hyena and wild dog after DNA extraction with SSH (method 5). Only the results from lane 17 in figure 3.8 was used for cloning and sequencing as it was visible under 70% UV light for excision from the gel.

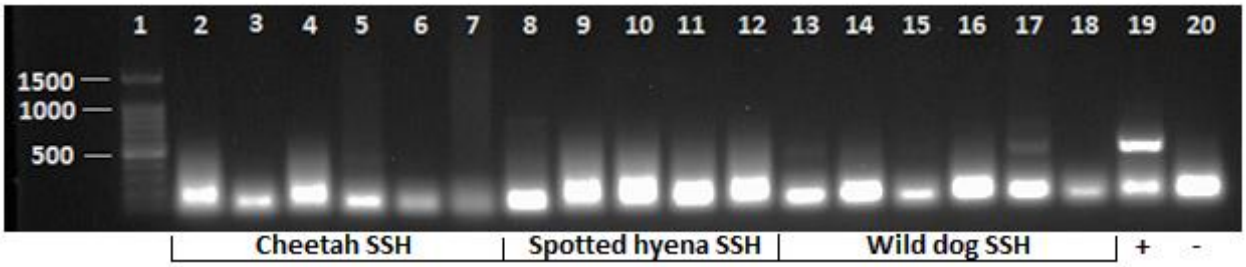


Figure 3.8: Agarose gel where only simplified sieving with bead homogenization (SSH) was used on three of the five wild carnivores. Lane 1, 100bp ladder; lane 19, positive control; lane 20, negative control.

Figure 3.9 shows the results for DNA extraction and PCR using lion faecal samples. Only the SSH (method 5) method was used for the lion samples. All the lion samples had positive amplification but only lane 4 and 6 were used for cloning and sequencing since they were the most visible under UV light. Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained.

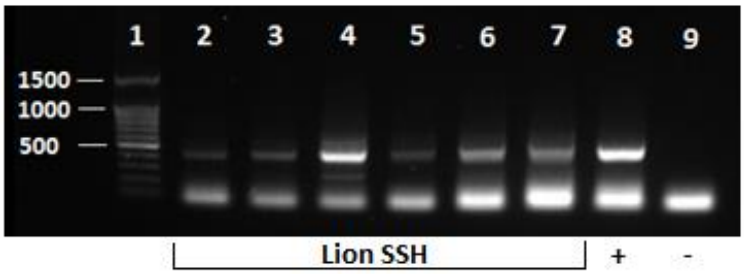


Figure 3.9: Agarose gel where only simplified sieving with bead homogenization (SSH) was used on the lion samples. Lane 1, 100 bp ladder; lane 2-7, lion faecal samples; lane 8 positive control; lane 9, negative control.

The primers used, namely JB3 and JB 4.5, were verified to be sufficiently conserved among a rage of nematodes (Hu *et al.* 2002). Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained. None of the sequences for the various wild carnivores were from *S. lupi.* The striped hyena results shows infection with roundworm as well as two nematode species. The results from the cheetah indicates *Gongylonema pulchrum* as well as gastrointestinal bacteria. *G. pulchrum* is known to infect cattle (Baylis *et al* 1926.; Makouloutou *et al.* 2013) as well as humans (Jelinek and Löscher 1994). By

consuming raw cattle meat the wild carnivores may have been infected with this parasite. Results from the lion indicated that flies can also be amplified with the JB3 and JB 4.5 primers. In the wild dog nematode, fungus and arthropod was amplified.

Faecal samples from black backed jackals that were stored in 70% ethanol at 4˚C over a period of a few years was used in DNA extraction with methods 5 and 6 (SS and SSH). Some of the samples were completely dry because the ethanol had evaporated. These samples were not used to extract DNA. A total of ten samples were randomly selected and used for DNA extraction. Black-backed jackals have previously been found with *S. lupi* infections therefore there was a high probability of finding *S. lupi* in faecal samples from these animals. Positive amplification was obtained for one of the jackal samples (lane 4 figure 3.10). The band was excised and cloned for sequencing. Table 3.5 shows a summary of the NCBI BLAST results of the sequences obtained. No *S. lupi* was detected.

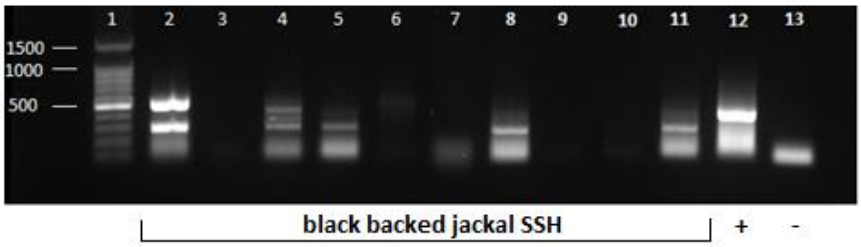


Figure 3.10: Agarose gel of PCR of black backed jackal samples that was stored in 70% ethanol. Lane 1, 100bp ladder; lane 12, positive control; lane 13, negative control.

Table 3.5: A summary of the NCBI BLAST results of DNA sequences from the cloned amplicons obtained from PCR of faecal samples from different wild and domestic carnivores.

Carnivore	NCBI Description	Short description	Query cover	E-Value	Identity	Accession
Dog (*Canis familiaris)*	*Gongylonema pulchrum* mitochondrial cox-1 gene for cytochrome c oxidase subunit I, partial cds	Nematode of the order Spirurida	83%	0	99%	AB513728.1
Striped hyena (*Hyaena hyaena*)	*Gongylonema pulchrum* mitochondrial cox-1 gene for cytochrome c oxidase subunit I, partial cds, isolate: Sato-036	Nematode of the order Spirurida	83%	0	99%	AB513728.1
	Bursaphelenchus conicaudatus mitochondrial CO1 gene for cytochrome oxidase subunit 1, partial cds, isolate:okinawa 1	Roundworm in the order Aphelenchida	98%	7.00E-148	89%	AB083728.1
	Panagrolaimus paetzoldi mitochondrial partial COI gene for cytochrome oxidase subunit 1, specimen voucher 13S17E9	Nematode of the order Rhabditida	89%	1.00E-150	92%	FN998934.1
	Neurospora crassa OR74A mitochondrion, complete genome	Red bread mould of the phylum Ascomycota	96%	5.00E-169	92%	KC683708.1
Cheetah (*Acinonyx jubatus*)	*Gongylonema pulchrum* mitochondrial cox-1 gene for cytochrome c oxidase subunit I, partial cds, isolate: Sato-036	Nematode of the order Spirurida	82%	3.00E-145	99%	AB513728.1
	Butyrivibrio proteoclasticus B316 chromosome 1, complete sequence	Gastrointestinal bacterium	20%	3.00E-22	95%	CP001810.1
	Ruminococcus torques L2-14 draft genome	Bacterium from the gut	15%	2.00E-09	87%	FP929055.1
Lion (*Panthera leo*)	*Scathophaga stercoraria* voucher NCSU:FLYTREE 187 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	Yellow dung fly	97%	1.00E-165	91%	GU208675.1

	Gongylonema pulchrum mitochondrial cox-1 gene for cytochrome c oxidase subunit I, partial cds, isolate: Sato-036	Nematode of the order Spirurida	82%	3.00E-145	99%	AB513728.1
	Neurospora crassa OR74A mitochondrion, complete genome	Red bread mould of the phylum Ascomycota	97%	1.00E-165	92%	KC683708.1
Wild dog	*Analges sp.* JS-2011 voucher Cris16aA cytochrome oxidase subunit 1 gene, partial cds; mitochondrial	Arthropod	88%	6.00E-99	84%	JF733991.1
	Arthroderma obtusum strain ATCC 42129 mitochondrion, complete genome	Fungus	98%	3.00E-131	87%	FJ385029.1
	Paragonimus mexicanus isolate PisoVer2 cytochrome c oxidase subunit 1 (CO1) gene, complete cds; mitochondrial	Nematode of the order Rhabditida	6%	2.00E-04	100%	KC562309.1
	Toxocara canis cytochrome c oxidase I mRNA, partial cds; mitochondrial gene for mitochondrial product	Roundworm of the order Ascaridida	11%	2.00E-14	98%	AF182297.3
Jackal	*Scathophaga stercoraria* voucher NCSU:FLYTREE 187 cytochrome oxidase subunit 1 (CO1) gene	Yellow dung fly	97%	2.00E-163	91%	KC192980.1
	Gongylonema pulchrum mitochondrial cox-1 gene for cytochrome c oxidase subunit I, partial cds	nematode of the order Spirurida	83%	0	99%	AB513728.1
	Neobellieria triplasia voucher E27 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Insect	99%	2.00E-153	89%	JQ807208.1

FUTURE WORK

Semi nested PCR

S. lupi DNA from adult worms from dogs were used in a nested PCR to determine if the primers will work on *S. lupi* DNA. The forward primer COIintF was designed from conserved cytochrome oxidase I (*cox1*) regions of *B. pahangi*, *B. malayi*, *D. repens* and *O. volvulus* sequences (CASIRAGHI *et al.* 2001). This nested PCR may help increase DNA yield when faecal DNA is amplified. The results from the gel electrophoresis analysis are shown in figure 3.11. The primers amplified the *S. lupi* DNA from the adult nematodes with the correct amplicon sizes. The DNA is clearly visible after the nested PCR (figure 3.11, lane 4 and 5) indicating a significant increase in DNA concentration. This method may therefore solve the problem of low DNA yields from faecal DNA extraction.

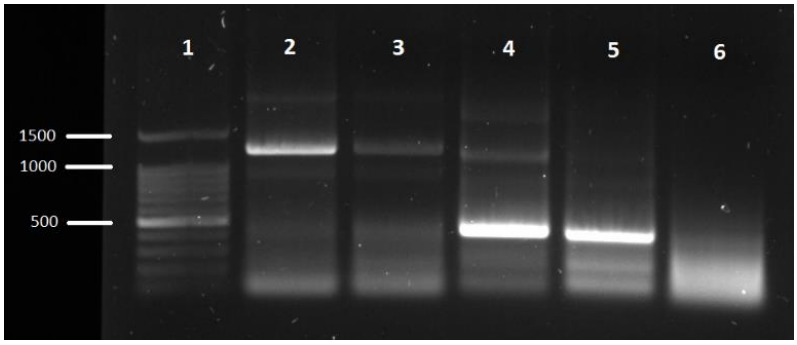


Figure 3.11: Gel electrophoresis results from semi-nested PCR. Lane 1 represents a 100bp ladder. Lane 2 and 3 represent the initial PCR with primers COIintF and JB4.5. Lane 4 and 5 represent the semi nested PCR with primers JB3 and JB4.5. Lane 6 is the negative control.

CONCLUSION

The aim of this study was to develop a non-invasive faecal DNA extraction method to screen wild carnivores for *S. lupi* infection while extracting DNA for future genetic analysis. The faecal DNA extraction methods tested were not successful in isolating *S. lupi* DNA although eggs were successfully separated from the faecal samples. Difficulties were encountered in successfully disrupting the *S. lupi* eggs without degrading the DNA. Some difficulties were also encountered with the consistency of the faecal samples. If the samples were too dry they could not be used in DNA extraction as the faeces could not be dissolved to be used in the various methods. *S. lupi* eggs were also not always present in the samples due to irregular egg shedding.

Some other nematodes were also detected during the DNA extraction process. Results also indicate possible nematode infection of *Bursaphelenchus conicaudatus, Panagrolaimus paetzoldi, Paragonimus mexicanus, Govgylonema pulchrum* and *Toxocara canis* in the wild carnivores. Although *S. lupi* DNA could not be isolated, these methods did work on these other nematode eggs. These methods may therefore work for other nematode species but at this stage could not be recommended for *S. lupi*. Unfortunately the methods described still require some modification for them to be viable and economical as a screening method. At this point we cannot recommend using faecal samples as a viable means to extract nematode DNA as its time consuming, expensive and seems to be lacking in successfully disrupting *S. lupi* eggs without degrading the DNA. Using faecal samples is also potentially dangerous as the samples may contain eggs from other zoonotic parasites.

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Chapter 4:

Screening *Spirocerca lupi* for *Wolbachia* and other endosymbionts

ABSTRACT

Wolbachia is an alphaproteobacterium that lives in an obligate symbiotic relationship with arthropods and nematodes. *Wolbachia* are cytoplasmically transmitted and found mostly within the reproductive organs of their symbionts. The *Wolbachia* genus can be divided into various clades known as supergroups. The supergroups C, D and F represent various nematode species as hosts for *Wolbachia*. It has been shown that antibiotics can be effective in treating filarial infections by eliminating the *Wolbachia* endosymbiont. Once the *Wolbachia* is eliminated the adult nematodes as well as the microfilariae die, since *Wolbachia* provides essential metabolites including riboflavin, heme, glutathione, glycolytic enzymes as well as other compounds necessary for the biosynthesis of purines and pyrimidines. *Wolbachia* is also involved in embryogenesis and growth of the filarial nematodes. *Wolbachia* can therefore provide a means to indirectly treat nematode infection of humans as well as many animal species.

Spirocerca lupi is a parasitic nematode of dogs and causes spirocercosis, a disease that could lead to cancer of the oesophagus. Various primers have been designed in order to screen for the different supergroups of *Wolbachia*. A bacterium species similar to that of *Comamonas* spp. was found in *S. lupi* isolates from a dog in Israel. After screening *Spirocerca lupi* isolated from dogs in South Africa, with various primers designed for different *Wolbachia* supergroups as well as primers for *Comamonas* spp. it was clear that this endosymbiotic bacterium is not present in *S. lupi* in South Africa. Antibiotic treatment would therefore not be a viable option in treating spirocercosis in local dogs.

INTRODUCTION

Discovery of *Wolbachia*

Wolbachia (class alphaproteobacteria) was first discovered in the ovaries of *Culex pipientis* (common house mosquito) in 1924 (HERTIG and WOLBACH 1924), but a complete description of *Wolbachia pipientis* was only published in 1936 (HERTIG 1936). Most insect species (25-70%) as well as many nematodes harbour an obligate intracellular bacterium of the genus *Wolbachia,* which is cytoplasmically transmitted (O'NEILL *et al.* 1997; WERREN 1997; STOUTHAMER *et al.* 1999; HOERAUF and RAO 2007). For nematodes, *Wolbachia* was first restricted to filarial nematodes of the family Onchocercidae which includes the animal and human parasites *Brugia malayi*, *Onchocerca volvulus* and *Wuchereria bancrofti* (FENN and BLAXTER 2006). A study of 21 non-filarial nematode species revealed that none harboured *Wolbachia* (BORDENSTEIN *et al.* 2003). Later a *Wolbachia*-like bacterium was discovered in the plant-parasitic nematode *Radopholus similis* (HAEGEMAN *et al.* 2009).

The effect of *Wolbachia* on the host

Nematodes transmit *Wolbachia* of the clades C, D and F transovarially as has been demonstrated though electron microscopy (HOERAUF and RAO 2007). In filarial nematodes *Wolbachia* is present in the lateral chords of the hypodermis in both males and females as well as the entire reproductive tract of females (KRAMER *et al.* 2003). These endobacteria are considered to be mutualists since all the adult nematodes of the infected species harbour the *Wolbachia* bacterium (WERREN *et al.* 2008). The *Wolbachia* are mutualistic endosymbionts required for host survival as well as embryogenesis of microfilariae. They also contribute to some of the inflammatory responses of filariae infections in vertebrate hosts (HOERAUF and RAO 2007). This has been demonstrated by treatments with antibiotics where the *Wolbachia* is eradicated and the host suffers delayed moulting, reduced growth rates, aberrant embryogenesis and eventually dies (CASIRAGHI *et al.* 2002; FENN and BLAXTER 2006). Since the genome of *Wolbachia* has been sequenced, careful annotation of the gene sequence also revealed that the *Wolbachia* can provide nematodes with essential metabolites. These metabolites include

riboflavin, heme, glutathione, glycolytic enzymes as well as other compounds necessary for the biosynthesis of purines and pyrimidines (FOSTER *et al.* 2005).

Phylogeny of *Wolbachia*

Bacterial isolates showing more than 3% nucleotide differences in their full length 16S rDNA can be attributed to different species. Since most *Wolbachia* supergroups have differences of around 3% they could potentially be elevated to species level (HOERAUF and RAO 2007). *Wolbachia* of filarial nematodes specifically has been proposed as a different species (PFARR *et al.* 2007). On the other hand recent phylogenetic studies revealed that nematode *Wolbachia* are not monophyletic (BORDENSTEIN *et al.* 2009). Also of interest, the phylogeny of *Wolbachia* from filarial nematodes can be matched to their hosts which is a clear indication of a long term co-evolution of these species (CASIRAGHI *et al.* 2001).

The genus *Wolbachia* is divided into supergroups (CASIRAGHI *et al.* 2005) encompassing symbionts of arthropods (A, B) (WERREN *et al.* 1995) and filarial nematodes (C and D) (BANDI *et al.* 1998). Supergroup E is *Wolbachia* from springtails (Collembola) (VANDEKERCKHOVE *et al.* 1999). The supergroup F are found in various species such as arthropods (termites) and filarial nematodes (*Mansonella spp.*) and scorpions (CASIRAGHI *et al.* 2001; LO *et al.* 2002; RASGON and SCOTT 2004; BALDO *et al.* 2007; HOERAUF and RAO 2007; KEISER *et al.* 2008). Two relatively new supergroups include the *Wolbachia* from Australian spiders (group G) (ROWLEY *et al.* 2004) and Isopteran species (group H) (BORDENSTEIN and ROSENGAUS 2005; HOERAUF and RAO 2007).

An approximation of the genus *Wolbachia* is depicted in Figure 4.1 (HOERAUF and RAO 2007). Further genetic diversity has been discovered in *Ctenocephalides felis* (cat flea) (GORHAM *et al.* 2003) and *Dipetalonema gracile* (filarial nematode) (CASIRAGHI *et al.* 2005) although these two groups have not yet been classified into a supergroup (HOERAUF and RAO 2007). It was suggested that *Wolbachia* from *D. gracile* be placed in the supergroup C and new supergroups P and Q were also suggested for the *Wolbachia* from quill mites (GLOWSKA *et al.* 2015). A more recent maximum likelihood construction of the *Wolbachia* supergoup phylogeny is shown in figure 4.2 as it was presented by Glowska *et al.* (2015).

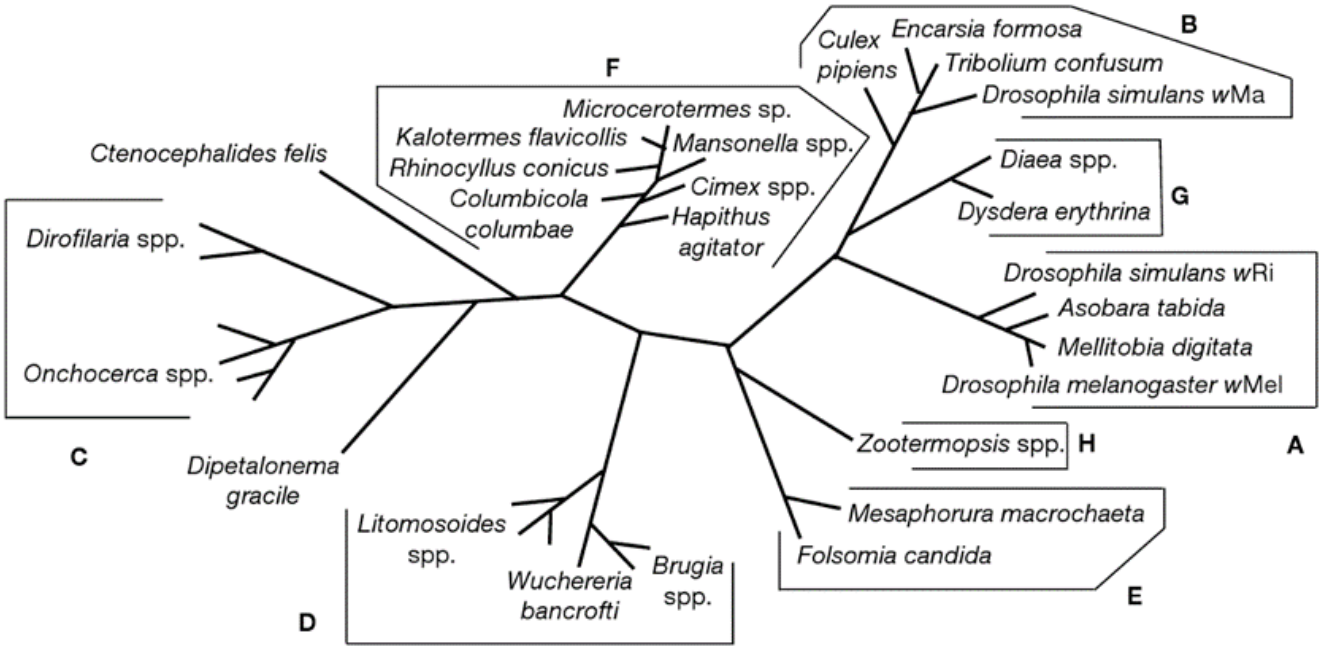


Figure 4.1: Unrooted tree of the main *Wolbachia* supergroups as compiled by Hoerauf and Roa (2007) from various sources. It is an approximation of the true tree and the branch lengths do not represent evolutionary distance. The *Wolbachia* from the filarial nematode *D. gracile* and arthropod *C. felis* have not yet been grouped into specific supergroups but show significant molecular diversity.

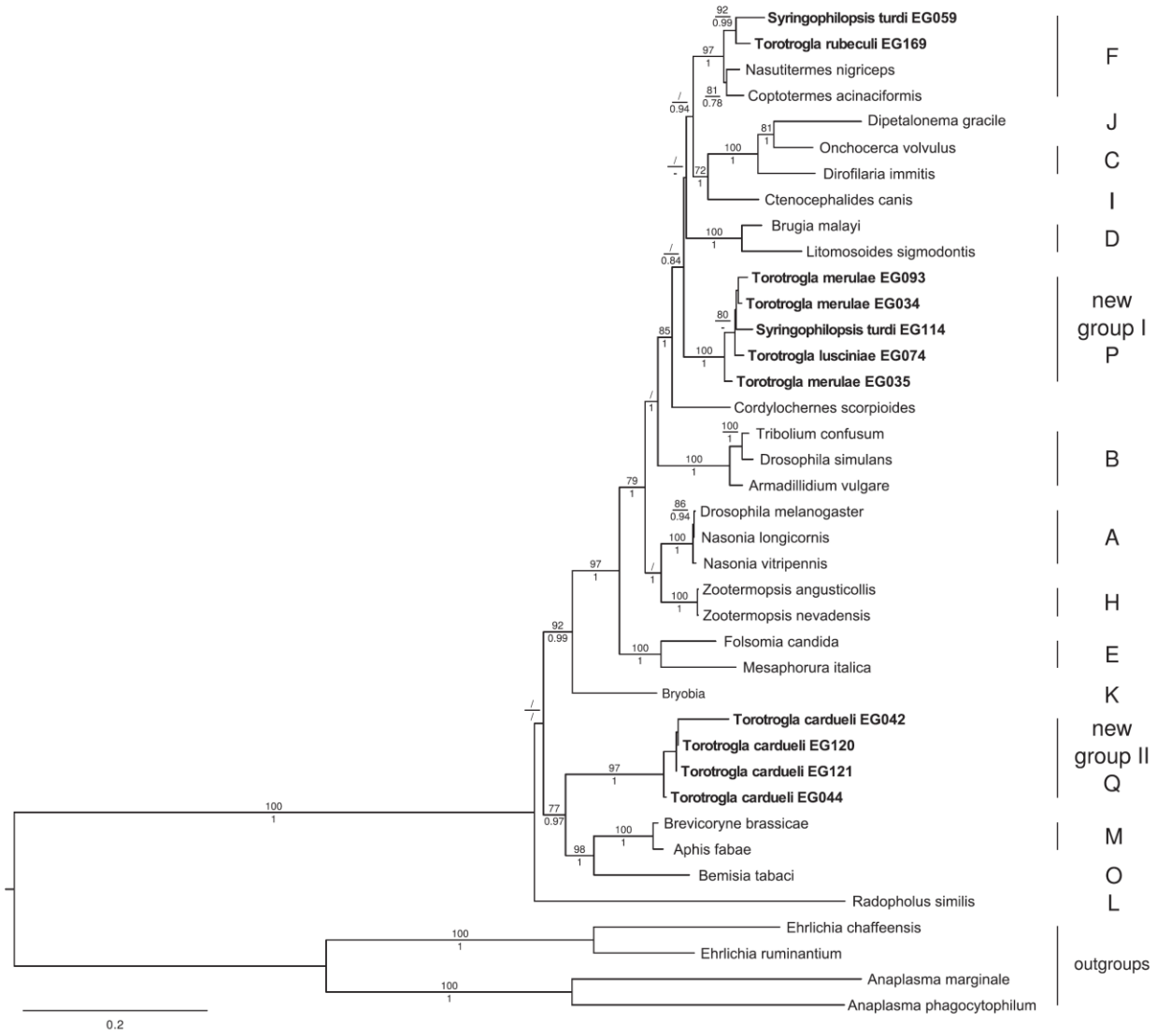


Figure 4.2: A maximum likelihood reconstruction of *Wolbachia* supergroups based on a supermatrix composed of 5 bacterial loci namely 16S *rRNA, coxA, ftsZ, gltA,* and *groEL* (GLOWSKA *et al.* 2015). The quill mites are highlighted in bold as this was the species of interest in the study by Glowska *et al.* (2015). Numbers on clades represent bootstrap support for 1000 replicates and posterior probabilities from bayesian analysis (below). Only values above 70% bayesian or 0.7 posterior probability are shown. Lower values are represented by /.

Wolbachia in filarial nematodes

Filarial nematodes (or filariae) are thread like parasitic roundworms that are transmitted by arthropod vectors. These nematodes cause major health diseases among humans as well as other animals in many tropical and subtropical areas. The disease produced by these nematodes depends on the tissues where the adults and microfilariae reside (BARON 1996). If they reside in the lymph vessels, as with *Wuchereria bancrofti* and *Brugia malayi*, the vessels become blocked and the hosts immune response causes lymphatic inflammation and dysfunction. This eventually leads to lymphedema and fibrosis (BARON 1996). Onchocerciasis is caused by the filarial worm *Onchocerca volvulus*. These nematodes migrate through the skin and connective tissues and cause skin pigmentations and dermatitis which leads to skin atrophy and loss of skin elasticity. If the microfilaria migrate to the eye, blindness can result. *Dirofilaria immitis* is a filarial parasite of dogs which resides in the heart (BARON 1996).

Antibiotics have been shown to be effective in treating filarial infections by eliminating the *Wolbachia* endosymbiont present in the filarial nematodes. For example oxytetracycline was tested *in vivo* against *Onchocerca ochengi* in a small population of cattle. All adult worms as well as the microfilariae were killed and the intradermal nodules were completely resolved nine months after initial treatment (LANGWORTHY *et al.* 2000). It has also been shown that tetracycline treatment is very effective in blocking embryo development of *Brugia pahangi* and *Dirofilaria immitis* (BANDI *et al.* 1999). Anti-*Wolbachia* therapy through antibiotics can deliver safe macrofilaricidal activity without long term treatment as well as avoiding the risk of severe adverse events such as in the case of treatment with ivermectin (TAYLOR *et al.* 2005, 2010). The aim of this project is to identify any endosymbiotic bacteria present in *S. lupi* focusing specifically on *Wolbachia*, as it can potentially be used to indirectly treat spirocercosis.

MATERIALS AND METHODS

Nematode Samples

Adult *S. lupi* nematodes were obtained from the Onderstepoort Veterinary Academic hospital between 2005 and 2007. The nematodes were removed from dogs presenting with spirocercosis post mortem or after death from internal haemorrhaging. The nematodes were stored in 70% ethanol at 4°C.

DNA Extraction

Adult nematodes were washed three times in 96% ethanol in order to remove any residual blood or tissue. Once washed the nematodes were crushed in liquid nitrogen. DNA extraction was performed on each nematode using the DNeasy blood and tissue kit (Qiagen). The protocol for the purification of total DNA from animal tissue was followed according to the manufacturers instructions.

Polymerase chain reaction

Table 4.1 lists the various primers used in the study and amplification conditions are described in table 4.2. All reactions were performed with the GoTaq G2 Flexi DNA polymerase (Promega). Reaction mixtures consisted of 1x Flexi buffer, 2 mM of $MgCl_2$ and 1 unit taq from the GoTaq kit. The dNTP and primer concentrations differ for each primer set in accordance with the original authors, these are shown in table 4.2. Approximately 100 ng of adult *S. lupi* DNA was used. All reactions were made up to a final volume of 25 µl with ultra-high quality water. The thermal profiles of the various primers are also shown in table 4.2.

Table 4.1: Primer summary for quick reference

Primers	Short description	Amplicon	Reference
99F and 994R	Amplifies 16S rRNA of *W. pipientis*	895 bp	(O'NEILL *et al.* 1992; AIKAWA *et al.* 2009)
ComF323 and ComR1393	Target *rrs* gene of 16S rRNA of *Comamonas* spp.	1000 bp	(GOTTLIEB *et al.* 2012)
ComNest F and ComR1393	Target *rrs* gene of 16S rRNA of *Comamonas* spp.	600bp	(GOTTLIEB *et al.* 2012)
fD1 and rD1	Amplification of 16S rDNA, amplify a wide variety of bacterial taxa	1500 bp	(WEISBURG *et al.* 1991)
fD1 and rP2	Amplification of 16S rDNA, amplify most eubacteria	1500 bp	(WEISBURG *et al.* 1991)
Fts3F and fts5R	Amplify a segment of the *ftsZ* gene	750 bp	(KONDO *et al.* 2002a; AIKAWA *et al.* 2009)
Wolb Fftsz and Wolb Rftsz	Species specific amplification based on conserved sequences of *Wolbachia ftsZ* gene	550 bp	(ROSSI *et al.* 2010)
Wsp 81F and wsp 691R	General *wsp* primers, designed to detect A and B strain arthropod *Wolbachia*	590-632 bp	(ZHOU *et al.* 1998)
WSPestF and WSPestR	Targets entire *wsp* gene designed against conserved portions of non-coding regions of the bacterium	700 bp	(BAZZOCCHI *et al.* 2000)
WSPintF and WSPintR	General primers for amplifying portions of the *wsp* gene of the C and D strain *Wolbachia*	590 bp	(BAZZOCCHI *et al.* 2000)

Table 4.2: Reaction mixtures and thermal profiles of each primer set.

Primers	Reaction mixture	Thermal profile
99F (5' – TTG TAG CCT GCT ATG GTA TAA CT) **994R** (5' – GAA TAG GTA TGA TTT TCA TGT)	0.2 mM of each dNTPs 400 nM of each primer	Initial denaturation of 5 min at 95°C followed by 30 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1. Final elongation of 5 min at 72°C
ComF323 (5' – CCT CGG GTT GTA AAC TGC TT) **ComR1393** (5' – TCT CTT TCG AGC ACG AAT CC) **ComNest F (not optimized)** (5' – ACT GCC ATT GTG ACT GCA AG)	0.2mM of each dNTPs 1 µM of each primer	Initial denaturation of 3 min at 95°C followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. Final elongation of 5 min at 72°C
fD1 (5' – AGA GTT TGA TCC TGG CTC AG) **rD1** (5' – AAG GAG GTG ATC CAG CC) **rP2** (5' – ACG GCT ACC TTG TTA CGA CTT)	0.2 mM of each dNTPs 10 pmol of each primer	Initial denaturation of 5 min at 95°C followed by 35 cycles of 95°C for 2 min, 42°C for 30 sec and 72°C for 4 min. Final elongation of 20 min at 72°C
fts3F (5' – GTA TGC CGA TTG CAG AGC TTG) **fts5R** (5' – GCC ATG AGT ATT CAC TTG GCT)	0.2 mM of each dNTPs 4 µM of each primer	Initial denaturation of 2 min at 94°C followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Final elongation of 5 min at 72°C
Wolb Fftsz (5' – ATA ACA GCA GGA ATG GGT GGT) **Wolb Rftsz** (5' – TCA CGC ACT CTA TTT GCT GCA)	0.2 mM of each dNTPs 7.2 pmol of each primer	Initial denaturation of 2 min at 94°C followed by 40 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 30 sec. Final elongation of 10 min at 72°C
***wsp* 81F** (5' – TGG TCC AAT AAG TGA TGA AGA AAC) ***wsp* 691R** (5' –AAA AAT TAA ACG CTA CTC CA)	0.2 mM of each dNTPs 4µM of each primer	Initial denaturation of 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Final elongation of 5 min at 72°C
WSPestF (5' – TTA GAC TGC TAA AGT GGA ATT) **WSPestR** (5' – AAA CCA CTG GGA TAA CAA GA)	0.2 mM of each dNTPs 1 µM of each primer	Initial denaturation of 5 min at 94°C followed by 35 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 90 sec. Final elongation of 5 min at 72°C
WSPintF (5' –TAG (CT) TAC TAC ATT CGC TTG CA) **WSPintR** (5' – CCA A(CT) AGT GC (CT) ATA AAG AAC)	0.2 mM of each dNTPs 1 µM of each primer	Initial denaturation step of 5 min at 95°C followed by 35 cycles of 95°C for 45 sec, 50°C for 45 sec and 72°C for 90 sec. Final elongation of 5 min at 72°C

Polymerase chain reaction optimization

After the initial polymerase chain reactions, optimizations were performed for all the various primers. The $MgCl_2$ concentrations were set to range from 2 mM to 8 mM with four different annealing temperatures as shown in table 4.3. This gave a total of 16 different reactions for each primer set.

Table 4.3: Annealing temperature range for the optimization of the various primers

Primers	**$MgCl_2$ range**	**Annealing temperatures range**	**Primers**	**$MgCl_2$ range**	**Annealing temperatures range**
99F and 994R	2 mM 4 mM 6 mM 8 mM	48 °C 50 °C 52 °C 54 °C	**Wolb Fftsz and Wolb Rftsz**	2 mM 4 mM 6 mM 8 mM	56 °C 58 °C 60 °C 62 °C
ComF323 and ComR1393	2 mM 4 mM 6 mM 8 mM	54 °C 56 °C 58 °C 60 °C	**Wsp 81F and Wsp 691R**	2 mM 4 mM 6 mM 8 mM	50 °C 52 °C 55 °C 57 °C
fD1, rD1 and rP2	2 mM 4 mM 6 mM 8 mM	40 °C 42 °C 44 °C 46 °C	**WSPestF and WSPestR**	2 mM 4 mM 6 mM 8 mM	48 °C 50 °C 52 °C 54 °C
Fts3F and fts5R	2 mM 4 mM 6 mM 8 mM	50 °C 52 °C 55 °C 57 °C	**WSPintF and WSPintR**	2 mM 4 mM 6 mM 8 mM	46 °C 48 °C 50 °C 52 °C

Agarose gel electrophoresis analysis and PCR purification

PCR reactions were analysed by gel electrophoresis using an agarose gel made with 1% agarose, 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and ethidium bromide at 25 ug/35 ml of

agarose gel. All analyses were performed at 100V for approximately 40 min to ensure adequate separation. Gels were examined under 70% UV light and positive amplicons were excised from the agarose gel. These were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturers instructions.

Cloning

Cloning was performed using the pGEM-T Easy vector system (Promega) according to the manufacturers instructions. JM109 High Efficiency Competent Cells were used with this vector system as was recommended by the manufacturers. Ligation reactions were incubated overnight at 4°C to ensure adequate ligation before transformation was performed. Cells were plated on Luria-Bertani medium supplemented with 100 µg/ml ampicillin, 50 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Blue-white screening was used for selection of recombinants. Selected colonies were grown overnight at 37˚C in liquid cultures consisting of liquid Luria-Bertani medium supplemented with 100 µg/ml ampicillin

Miniprep and Restriction enzyme digestion

Small DNA preparations were performed on the liquid cultures using the QIAprep spin miniprep kit (Qiagen) with the centrifugation protocol according to manufacturers instructions. Restriction digestion was performed with *Eco*RI (Promega) and approximately 100 ng template DNA. The reaction was incubated for 90 min at 37°C before gel electrophoresis analysis was performed to determine if the correct inserts were present.

RESULTS AND DISCUSSION

Wolbachia can be used as a means to control parasitic infections of nematodes as it is an obligate endosymbiont required for embryogenesis as well as host survival. To determine if *S. lupi* contains the endosymbiotic bacterium *Wolbachia*, various primers specific for different species and genes of *Wolbachia* as well as other edosymbiotic eubacteria were used to screen for any endosymbiotic bacteria present in *S. lupi*. The results for the various primers used are described below.

Primers 99F and 994R

These primers were designed to be specific for *Wolbchia pipientis* 16S rRNA gene where the forward primer is in the variable V1 region and corresponds to *Escherichia coli* position 79-99 forward and the reverse primer which is the reverse compliment of the variable V6 region and corresponds to *E. coli* position 1012-994 reverse (O'NEILL *et al.* 1992). The amplicon was expected to be approximately 895 bp. There was no positive amplification with *S. lupi* adult DNA as can be seen in figure 4.3. No contamination was evident in the negative control.

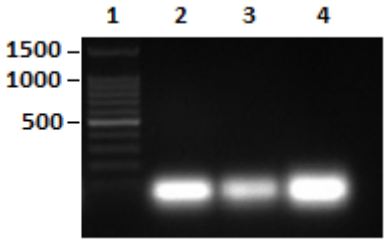


Figure 4.3: Agarose gel electrophoresis analysis of amplification with primers 99F and 994R. Lane 1 represents a 100 bp ladder (Promega). Lane 2 and 3 shows PCR product from *S. lupi* adult worms. Lane 4 shows the negative control.

The PCR reaction for primers 99F and 994R was optimized. The results for the optimization reaction are shown in figures A-D in figure 4.4. There seems to be some indication of amplification with band sizes of 500 and smaller but nothing in the order of 895 bp as was expected for this primer set.

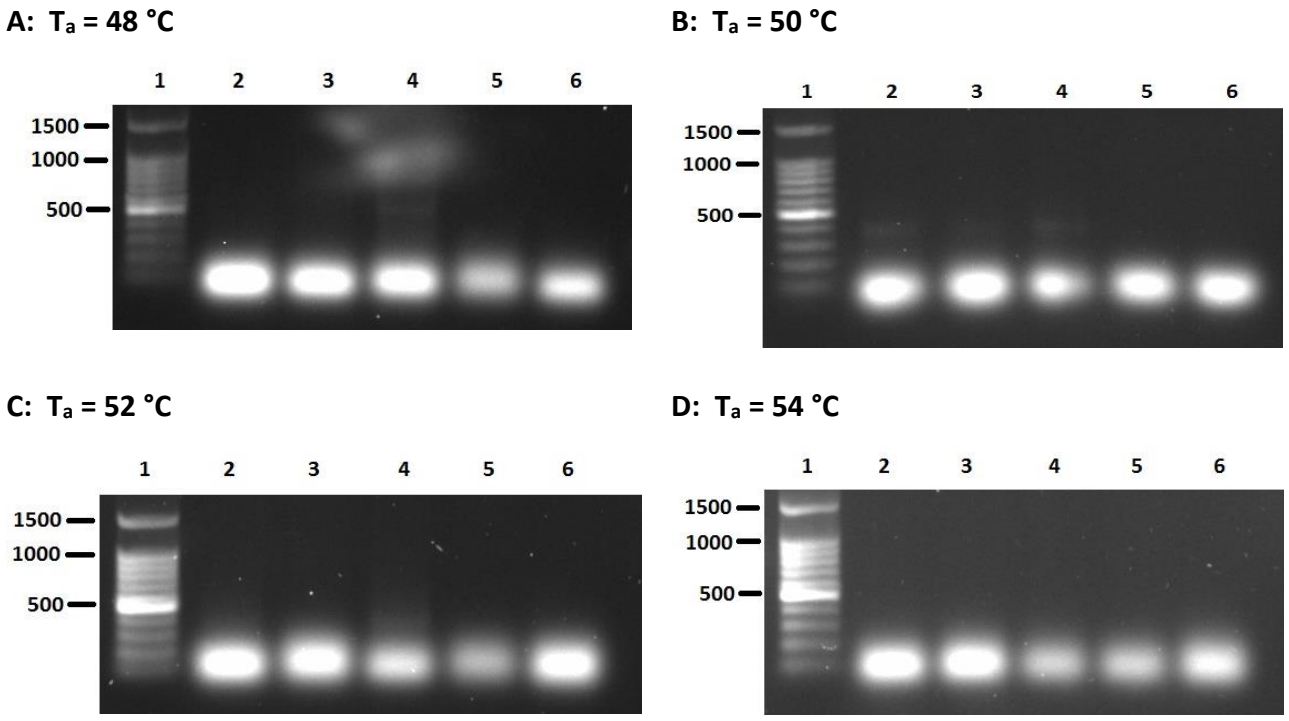


Figure 4.4: Agarose gel analysis of the optimization of primers 99F and 994R. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

Primers ComF323, ComR1393 and ComNestF

These primers were based on a consensus sequence for *Comamonas spp.* (GOTTLIEB *et al.* 2012). A 1000 bp PCR product was expected but there was no clear amplification of a 1000 bp for any of the samples as can be seen in figure 4.5. Optimization was performed with these primers as with the previous primer set. The optimization results are shown in figure 4.6. No positive controls were used for this PCR as we did not have *Comamonas* DNA available. No amplification in the region of 1000 bp was seen on any of the electrophoresis gels.

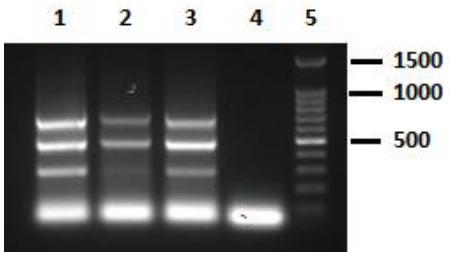
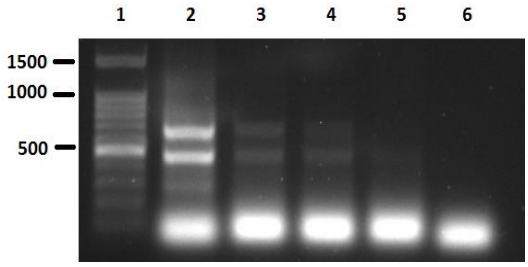
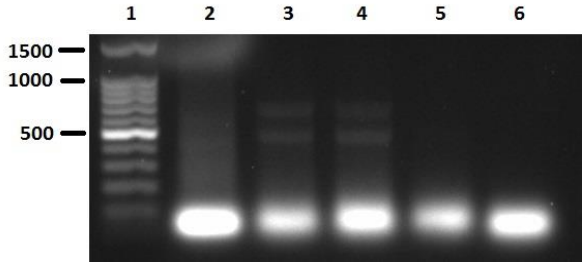


Figure 4.5: Agarose gel analysis of PCR amplification for primers ComF323 and ComR1393. Lane 1-3 represent PCRs performed with adult *S. lupi* DNA extracts. Lane 4 is the negative control and lane 5 is the 100 bp ladder.

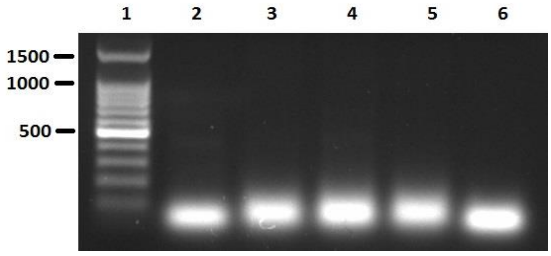
A: T_a = 54 °C



B: T_a = 56 °C



C: T_a = 58 °C



D: T_a = 60 °C

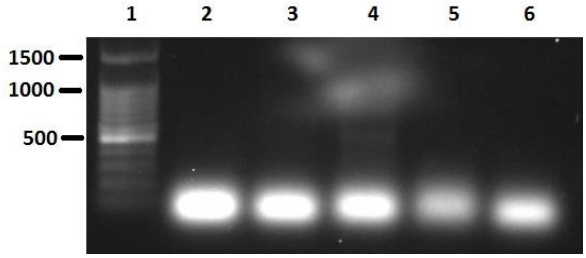


Figure 4.6: Agarose gel electrophoresis analysis of the optimization of primers ComF323 and ComR1393. For all figures lane 1 represents a 100 bp ladder. Lane 2 up till 5 represent $MgCl_2$ concentrations of 2 mM, 4 mM, 6 mM and 8 mM. Lane 6 represents the negative control. Annealing temperature are shown above each figure.

Gottlieb *et al*. (2012) used the ComNestF primer to replace the forward primer in order to perform a semi-nested PCR for which a 600 bp product was obtained. This primer configuration was tested using the PCR conditions that obtained the best results during optimization of the original primer set namely

an annealing temperature of 54 °C with 2 mM $MgCl_2$. The results for this PCR are shown in figure 4.7. A 600 bp band can be seen in lanes 5 and 6. These amplicons were excised and cloned before sequencing. The sequences were compared to other sequences on the NCBI database through a BLAST search. No significant similarity was found to any sequences on the database, therefore these sequences do not originate from *Comamonas spp.*

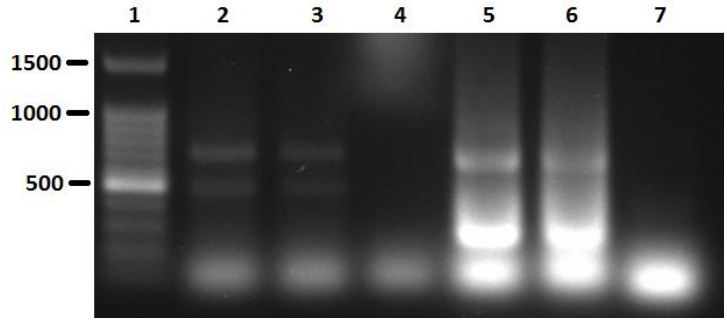


Figure 4.7: Agarose gel electrophoresis analysis of the nested PCR as described by Gottlieb *et al*. (2012). Lane 1 represents a 100 bp ladder. Lane 2 and 3 are PCR results for primers ComF323 and ComR1393 with adult *S. lupi* DNA. Lane 5 and 6 represents PCR results from primers ComNestF and ComR1393 with adult *S. lupi* DNA. Lanes 4 and 7 represent negative controls for these two PCRs.

Primers fD1, rD1 and rP2

Primers fD1, rD1 and rP2 were designed for the amplification of 16S rDNA (WEISBURG *et al.* 1991). The primer pair fD1 and rD1 designed to amplify a wide variety of bacterial taxa whereas the fD1 and rP2 primer pair are expected to amplify most eubacteria. The PCR product was expected to be in the order of 1500 bp. Initial amplification results can be seen in figure 4.8 with primers fD1 and rD1 on the left and fD1 and rP2 on the right. Primers fD1 and rP2 produced no amplicons of 1500 bp. Primers fD1 and rD1 did have positive amplification at approximately 1400 bp. Amplification with these primers was optimized as can be seen in figures 4.9 and 4.10. There was no significant band visible at 1500 bp after optimization for primers fD1 and rD1, but there was amplification visible at 1500 bp for primers fD1 and rP2. Sequencing did not yield any viable information as the BLAST results only indicated no significant similarity found.

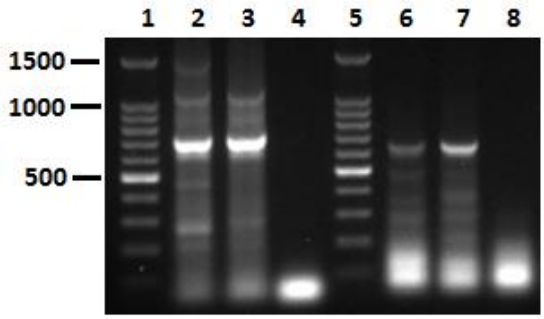


Figure 4.8: Agarose gel electrophoresis analysis of amplification with primers fD1, rD1 and rP2. Lane 1 and 5 represent 100 bp ladders. Lanes 2 and 3 are the amplification results for primers fD1 and rD1 with adult *S. lupi* DNA. Lanes 6 and 7 are the amplification results for primers fD1 and rP2 with adult *S. lupi* DNA. Lanes 4 and 8 represent negative controls.

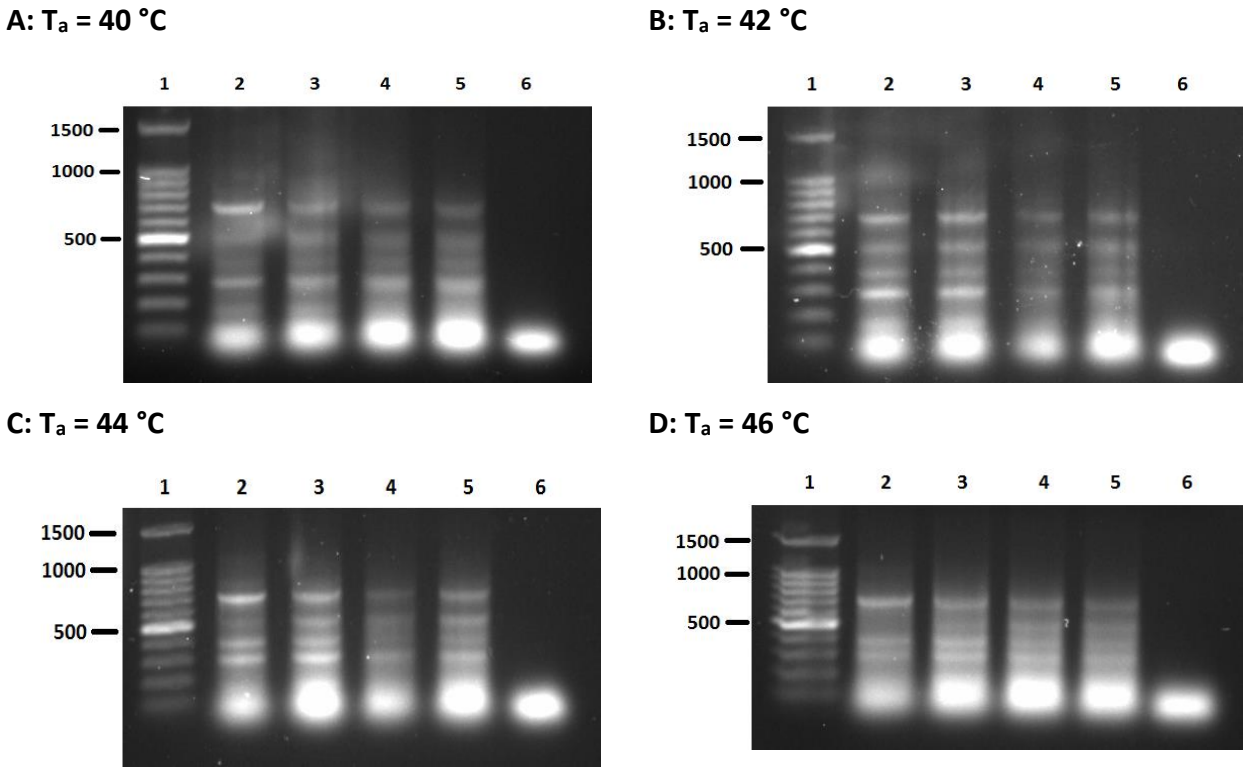


Figure 4.9: Agarose gel electrophoresis analysis of the optimization for primers fD1 and rD1. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

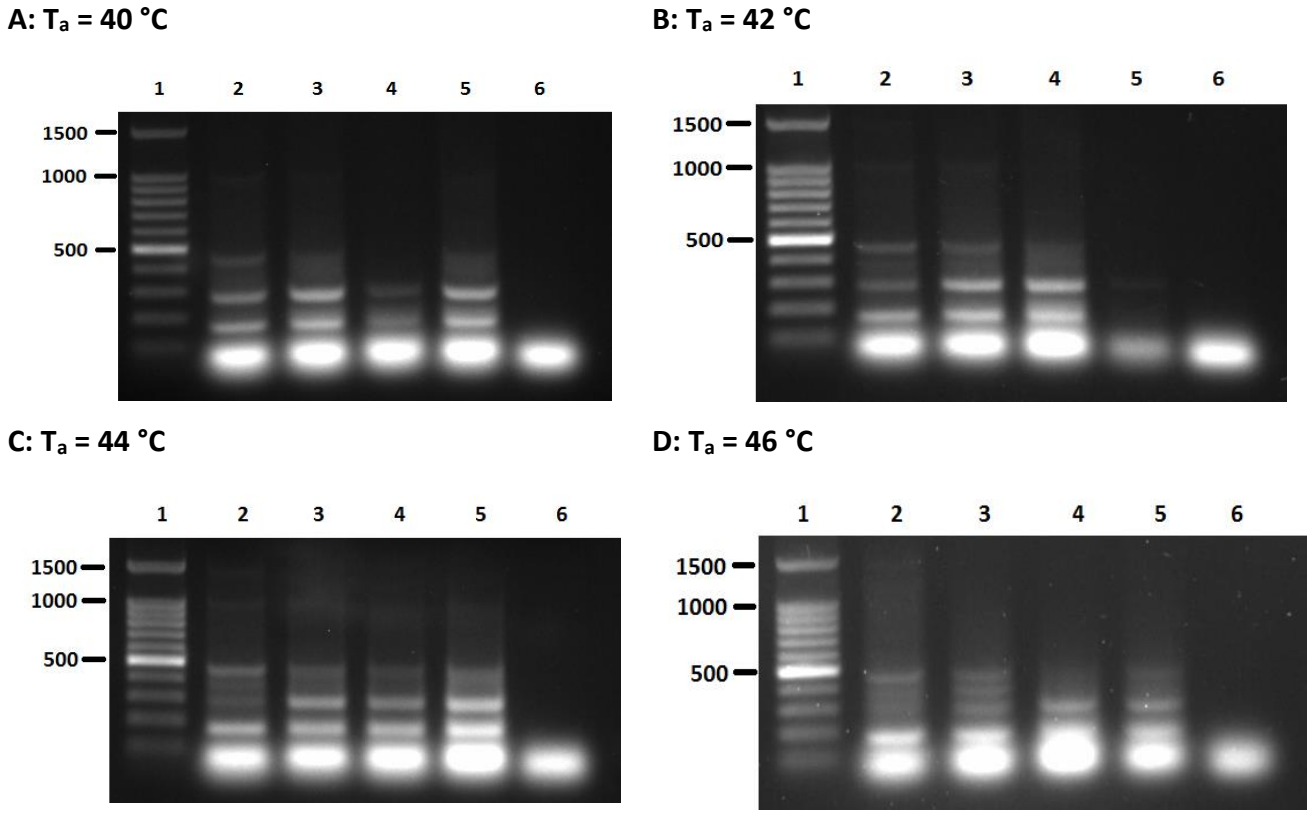


Figure 4.10: Agarose gel electrophoresis analysis of the optimization results for primers fD1 and rP2. For all figures lane 1 represents a 100 bp ladder. Lane 2 up till 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperature are shown above each figure.

Primers fts3F and fts5R

These primers were designed to amplify a segment of the ftsZ gene (KONDO *et al.* 2002b). An amplification product of approximately 750 bp was expected. Results for amplification of adult *S. lupi* DNA with these primers can be seen in figure 4.11. There was low levels of amplification at the 750 bp. Optimization was performed to see if higher amplification concentrations could be achieved. Optimizations are shown in figure 4.12. There were no significant results from any of the optimizations.

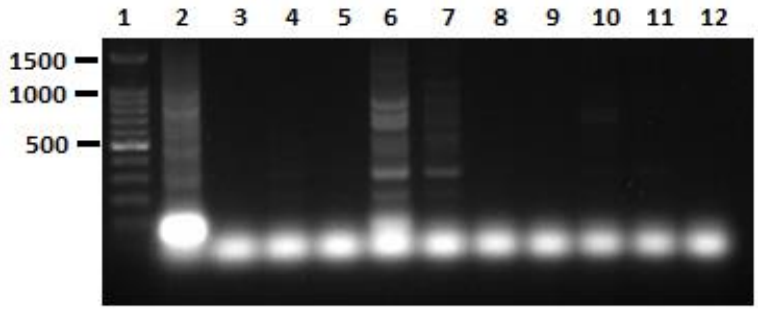


Figure 4.11: Agarose gel electrophoresis analysis for amplification of primers fts3F and fts5R. Lane 1 is a 100 bp ladder, lane 2 up to 11 are PCR products from ten adult *S. lupi* DNA samples. Lane 12 is the negative control.

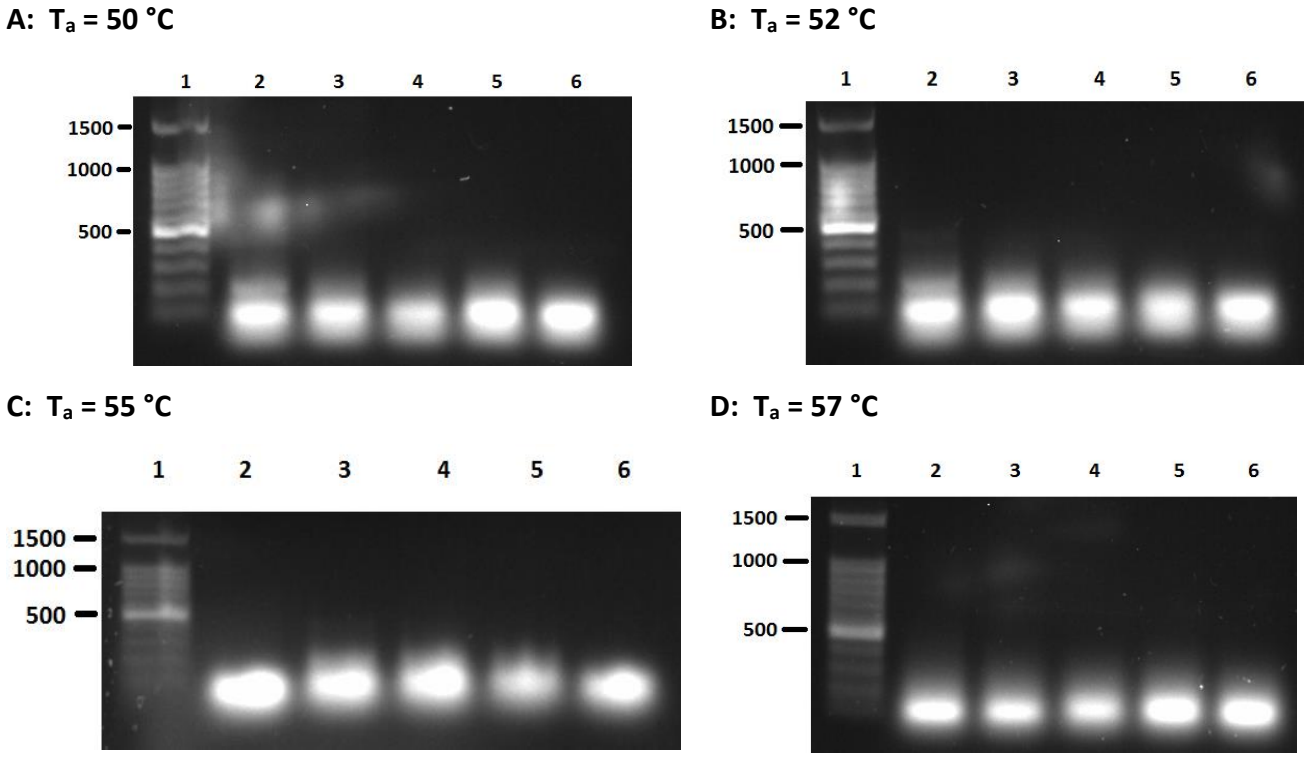


Figure 4.12: Agarose gel electrophoresis analysis of the optimization for primers fts3F and fts5R. For all figures lane 1 represents a 100 bp ladder. Lane 2 up till 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

Primers Wolb Fftsz and Wolb Rftsz

These primers were designed for species specific amplification based on conserved sequences of *Wolbachia* ftsZ gene (Rossi *et al.* 2010). An amplification product of approximately 550 bp was expected for these primers. There was no amplification for any of the *S. lupi* samples as can be seen in figure 4.13. These primers were also not used in further analysis. Optimizations are shown in figure 4.14. No amplification was obtained.

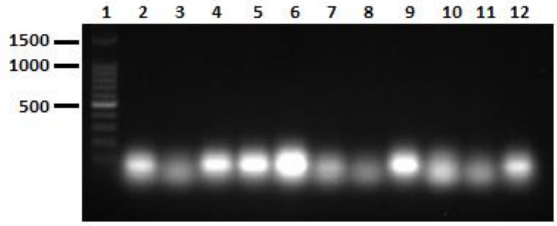
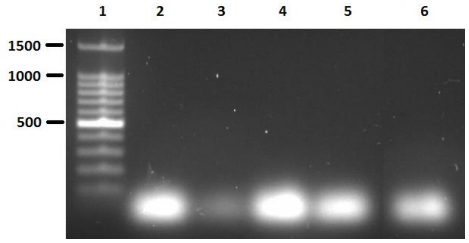
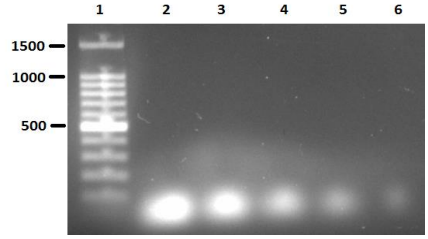


Figure 4.13: Agarose gel electrophoresis results for amplification with primers Wolb Fftsz and Wolb Rftsz. Lane 1 represents a 100 bp ladder, lane 2 through 11 are PCR results from ten adult *S. lupi* nematodes, and lane 12 is the negative control.

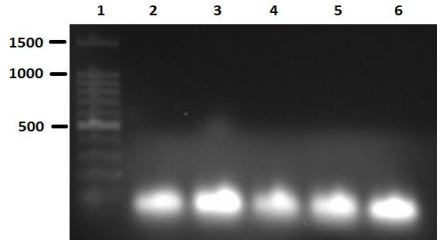
A: T_a = 56 °C



B: T_a = 58 °C



C: T_a = 60 °C



D: T_a = 62 °C

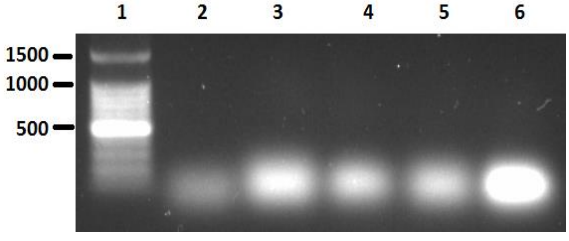


Figure 4.14: Agarose gel electrophoresis analysis for the optimization of primers Wolb Fftsz and Wolb Rftsz. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

Primers *wsp* 81F and *wsp* 691R

Primers *wsp* 81F and *wsp* 691R are general primers that can amplify the *wsp* gene fragment from a variety of *Wolbachia* strains (ZHOU *et al.* 1998). For these primers an amplicon of 590-632 bp was expected. The PCR results are shown in figure 4.15. For this specific PCR a fig wasp DNA sample was available. *Sycophaga sycomori* is a fig wasp that harbours *Wolbachia* and this sample was used as a positive control as can be seen in lane 6 of figure 4.15. None of the other samples had a positive amplification product corresponding to the positive control. Non-specific amplification was visible but this result could not be repeated in the PCR optimization as can be seen in figure 4.16. These amplicons were therefore not considered.

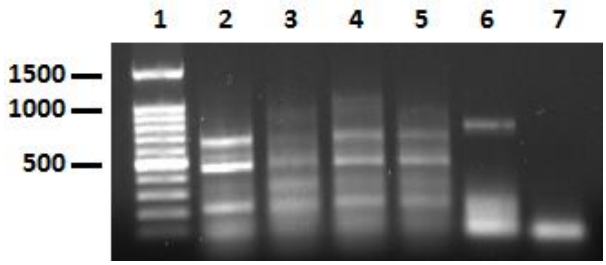


Figure 4.15: Agarose gel electrophoresis analysis for the amplification of primers *wsp* 81F and *wsp* 691R. Lane 1 represents a 100 bp ladder. Lane 2 up till 5 represents PCR results from adult *S. lupi* DNA. Lane 6 is a positive control from a fig wasp and lane 7 is a negative control.

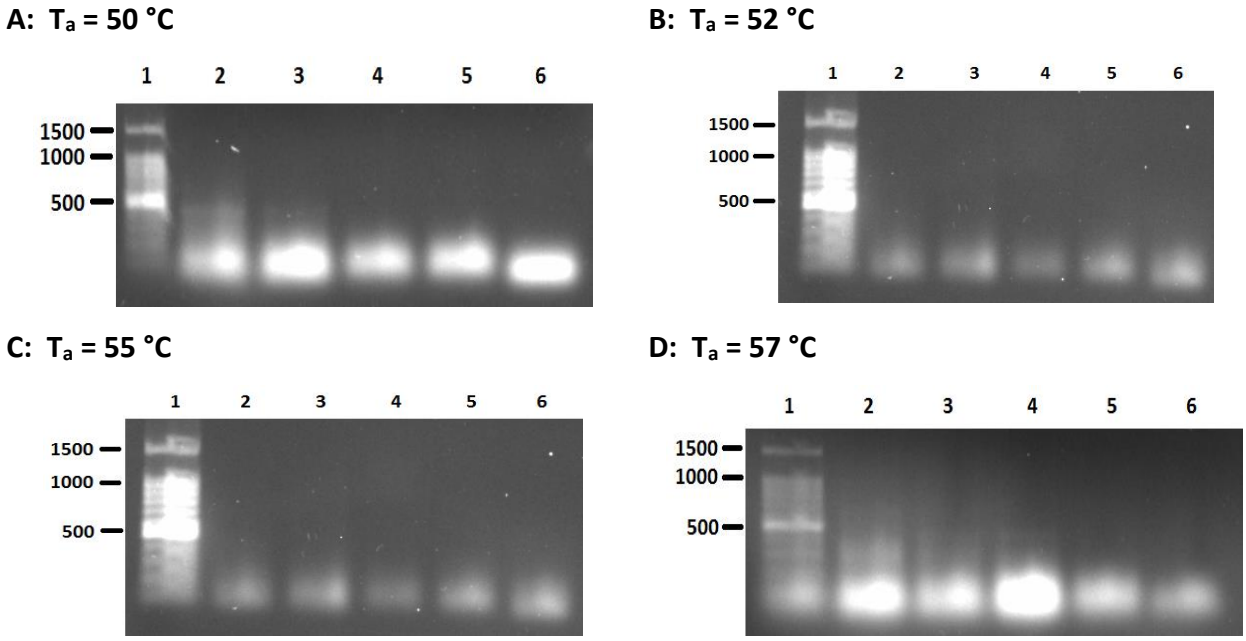


Figure 4.16: Agarose gel electrophoresis analysis for the optimization of primers *wsp* 81F and *wsp* 691R. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

Primers WSPestF and WSPestR

Primers WSPestF and WSPestR were designed on the basis of conserved portions of the non-coding regions of the arthropod *wsp* sequences available in genbank (BAZZOCCHI *et al.* 2000). These primers were used to obtain a complete *wsp* coding sequence for two filarial species *D. immitis* and *B. malayi*. The expected amplicon of approximately 700 bp was not observed in any of the *S. lupi* DNA samples as can be seen from the results in figure 4.17. Optimizations also yielded no improved results as can be seen in figure 4.18.

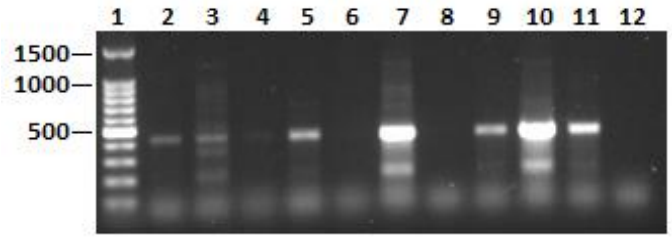
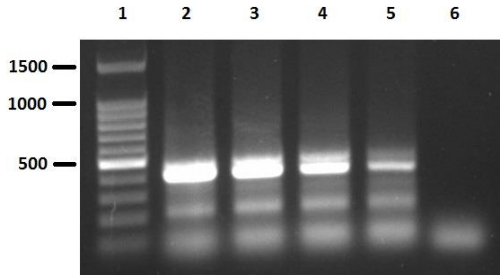
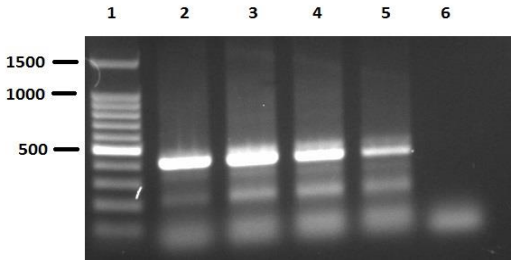


Figure 4.17: Agarose gel electrophoresis analysis for amplification with primers WSPestF and WSPestR. Lane 1 represents a 100 bp ladder. Lane 2 through till lane 11 represent ten adult *S. lupi* DNA samples. Lane 12 is the negative control.

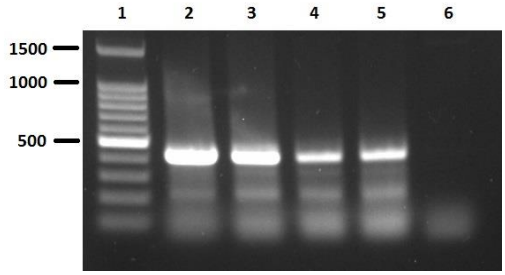
A: T_a = 48 °C



B: T_a = 50°C



C: T_a = 52 °C



D: T_a = 54 °C

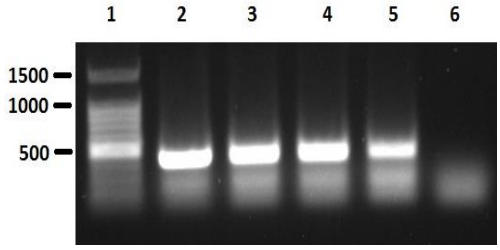


Figure 4.18: Agarose gel electrophoresis analysis of the optimization for primers WSPestF and WSPestR. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

Primers WSPintF and WSPintR

Primers WSPintF and WSPintR were designed on the basis of conserved sequences obtained from PCR with WSPestF and WSPestR primers (BAZZOCCHI *et al.* 2000). The results are shown in figure 4.19. An amplicon of approximately 590 bp was expected. There wasn't any clear amplicons that could be excised and cloned for sequencing corresponding approximately to the 600 bp marker. There was a PCR amplification product at approximately 500 bp size but this amplicon was not used for further analysis since PCR optimization did not yield similar results as can be seen in figure 4.20.

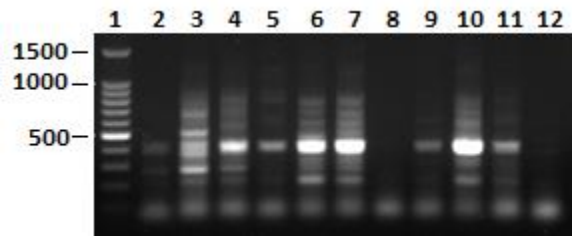


Figure 4.19: Agarose gel electrophoresis analysis for the amplification with primers WSPintF and WSPintR. Lane 1 represents a 100 bp ladder. Lane 2 through till lane 11 represents ten DNA samples from adult *S. lupi* nematodes. Lane 12 is the negative control.

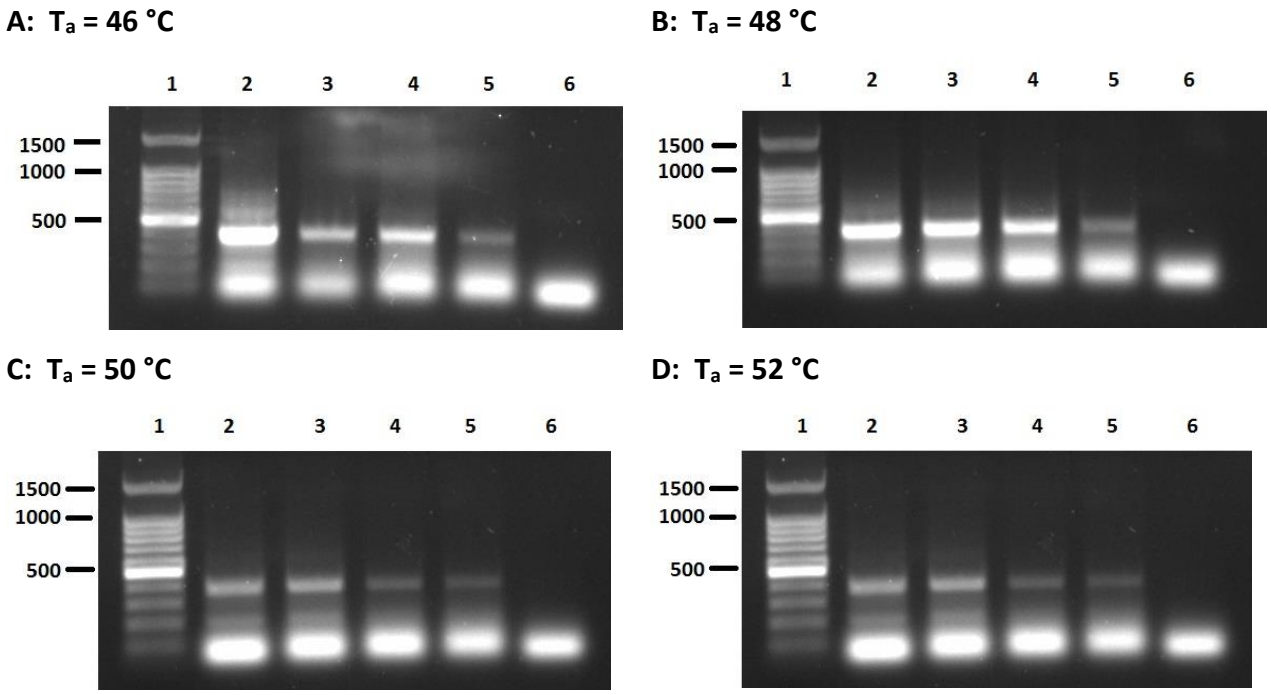


Figure 4.20: Agarose gel electrophoresis analysis for the optimization with primers WSPintF and WSPintR. For all figures lane 1 represents a 100 bp ladder. Lane 2 - 5 represent $MgCl_2$ concentrations of 2mM, 4mM, 6mM and 8mM. Lane 6 represents the negative control. Annealing temperatures are shown above each figure.

CONCLUSION

After screening with various primers designed to detect *Wolbachia* as well as other endosymbionts, *Spirocerca lupi* does not appear to have any of the tested endosymbiotic bacteria present. It was speculated that *Comamonas spp.* might be present in *S. lupi* from South Africa but current results indicate that it may not be since results from Gottlieb *et al.* (2012) could not be confirmed. *S. lupi* from South Africa does appear to be genetically distinct from those from Israel (as seen in chapter 2). Since there appears to be some genetic distinction between the regions, the *Comamonas spp* might have evolved in the Israeli nematodes but not in the South African variant. The amplification that was seen in the various electrophoresis results may be due to the fact that *S. lupi* is a gastrointestinal parasite, therefore common gastrointestinal bacteria are expected to be present on and within the nematode.

Antibiotic treatment would currently not be a viable means for treating spirocercosis, unless some endosymbiotic bacterium is present that has not yet been identified. Further analysis in this regard may be required to detect the presence of an as yet unknown bacterial endosymbiont.

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Chapter 5:

Concluding remarks

CONCLUDING REMARKS

The continued infringement of humans on wildlife borders increases the number of domestic dogs that come into contact with wild canids. This subsequently increases the risk of spillover of parasitic organisms from dogs to wild canids. In this study it was demonstrated that there is gene flow between domestic dogs and black backed jackals with coinciding *S. lupi cox1* haplotypes found in both species. This might pose a potential threat to the endangered African wild dog as well as other wild carnivores. Currently it is still unknown if the African wild dog is a host for *S. lupi*.

The genetic diversity of *S. lupi cox1* has increased with a number of new haplotypes identified that were not previously discovered during a study in domestic dogs in the Pretoria Metropole area (DE WAAL *et al.* 2012). This increase in genetic diversity could lead to an increase in anthelmintic resistance. There appears to be a genetic distinction between South African *S. lupi cox1* samples compared to other countries. This genetic diversity seems to indicate that there may be several cryptic species of *S. lupi*. Currently South African dogs and foxes in Denmark appear to host two of these cryptic species (AL-SABI *et al.* 2014). In order to classify the nematodes from these two countries as cryptic species a larger study with a larger region of the *cox1* gene as well as other genes need to be analysed and compared. DNA isolation of *S. lupi* from wolves might also shed some light on where this parasite originated from as well as its current species status, since it was first discovered in these canids.

In this study, it was shown that isolating DNA from canid faecal samples is not a viable alternative for non-invasive screening for *S. lupi* in wild canids. Egg shedding is inconsistent and unpredictable, faecal samples can be too dry or contain too much contaminating DNA from other species. The eggs themselves also proved too resilient and more often DNA was damaged due to excessive measures used to disrupt the eggs. In addition the primers used were designed from conserved regions of the *cox1* gene, therefore amplified various other nematode species as well as bacteria, fungi and fly DNA.

Spirocerca lupi adult nematodes can usually be obtained only through surgery, which is frequently not considered, or through necropsy. Since it is so difficult to obtain samples, especially from endangered species, some alternate method must be considered. A recent study indicated that *S. lupi* causes a unique immune response with increased plasma IL-8 concentrations in dogs with spirocercosis (DVIR *et al.* 2012). These immune indications along with further immune assays may lead to the development of a screening method. Such a method could be used for early diagnosis of *S. lupi* infection which could lead to treatment before the onset of cancer.

Alternative treatment of *S. lupi* may also be found in the existence of symbiotic endobacteria. The strategy involves the use of antibiotic treatment to kill essential endosymbiotic bacteria which would thereby indirectly kill the *S. lupi* nematodes. *S. lupi* isolates from Israel were shown to contain an endosymbiotic bacterium of the *Comamonas* species (GOTTLIEB *et al.* 2012). Unfortunately this same species could not be detected in *S. lupi* isolates from South Africa. In this study it was shown that there is some genetic diversity between isolates from South Africa compared to Israel, which might explain why these bacterium are not present in South African *S. lupi* nematodes. Since these bacteria do not appear to be present in *S. lupi* from South Africa this method of treatment is not currently a viable option.

Another aspect that has not been examined in any detail is the role of the intermediate hosts as well as paratenic hosts. Are these intermediate or paratenic hosts the primary agents causing the spread of the parasite, or do humans play a greater role in dispersion? Do the intermediate hosts or the paratenic hosts play a greater role in transmitting the parasite to the primary dog host? These question have not yet been explored and further studies are required to understand the full dynamics of the infective range of this parasite.

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