Diversity of *Spirocerca lupi* in domestic dogs and black-backed jackals *(Canis mesomelas)* from South Africa

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Highlights

- Evidence of transmission between *S. lupi* from domestic dogs to black-backed jackals or *vice versa*.
- Possible cryptic species of *S. lupi* in South Africa since there are indications of notable *cox1* genetic variation between *S. lupi* isolates from South Africa compared to Europe.
- Haplotypes are shared between domestic dogs and black-backed jackals.

Abstract

Spirocerca lupi is a parasitic nematode that causes spirocercosis predominantly in domestic dogs. *Spirocerca lupi* nematode samples were collected from four regions around South Africa and analyzed to compare the genetic diversity among the regions. A total of 56 *S. lupi* nematodes were obtained by necropsy from domestic dogs and wild black-backed jackals (*Canis mesomelas*). Sixteen different haplotypes of *cox1* were identified some of which are shared between regions as well as with black-backed jackal. The genetic similarity between *S. lupi* in domestic dogs and black-backed jackals indicates transmission between these canid species and may have potential conservation implications.

Keywords: Black-backed jackal, *Canis mesomelas, cox1,* genetic diversity, *Spirocerca lupi*

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). *Spirocerca lupi* is found in tropical and subtropical regions and may be fatal to its host (Van der Merwe et al., 2008; Elias et al., 2016). Coprophagous beetles serve as intermediate hosts for *S. lupi*. Birds, lizards and rodents may serve as additional paratenic

hosts for the L1 stage infective larvae (Bailey, 1972; Van der Merwe et al., 2008; Ravindran et al., 2014; Aroch et al., 2017). In a written *S. lupi* prevalence survey in South Africa, more cases of spirocercosis have been seen with 16% of the veterinarians considering spirocercosis to be a new phenomenon (Lobetti, 2014). In a similar study in Israel, spirocercosis has spread over time in spite of the preventative measures applied over the last three decades (Aroch et al., 2015).

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 for differentiation between closely related species (Avise et al., 1987; Moritz, 1994). A study in the Tshwane Metropole (Pretoria) area of South Africa indicated that the highest haplotype diversity was found within the domestic dog host (76%) compared to the diversity among hosts (28%) (de Waal et al., 2012). In the same study, 11 haplotypes were found among 60 adult nematodes from domestic dogs. In Denmark, a genetically distinct isolate of *S. lupi* was found in the red fox (*Vulpes vulpes*) (Al-Sabi et al., 2014). The aim of this study was to determine the haplotype genetic diversity of *S. lupi* in black-backed jackals compared to domestic dogs to determine if there is genetic transmission between the species.

Materials and methods

A total of 56 adult *S. lupi* nematodes were obtained from various regions of South Africa. Nine adult nematodes from four dogs from Durban in Kwa-Zulu Natal, 12 nematodes from six dogs from Grahamstown in the Eastern Cape, 28 nematodes from 21 dogs from the Tshwane Metropole in the Gauteng Province and seven immature adults (found in the gastric mucosa and arteries) from three black backed jackals from Kimberley in the Northern Cape (Figure 1). The nematodes were extracted at necropsy from dogs euthanized due to severe spirocercosis as well as jackals trapped by local farmers protecting their livestock. All nematodes were stored in 70% ethanol at 4 °C until DNA extraction was performed.

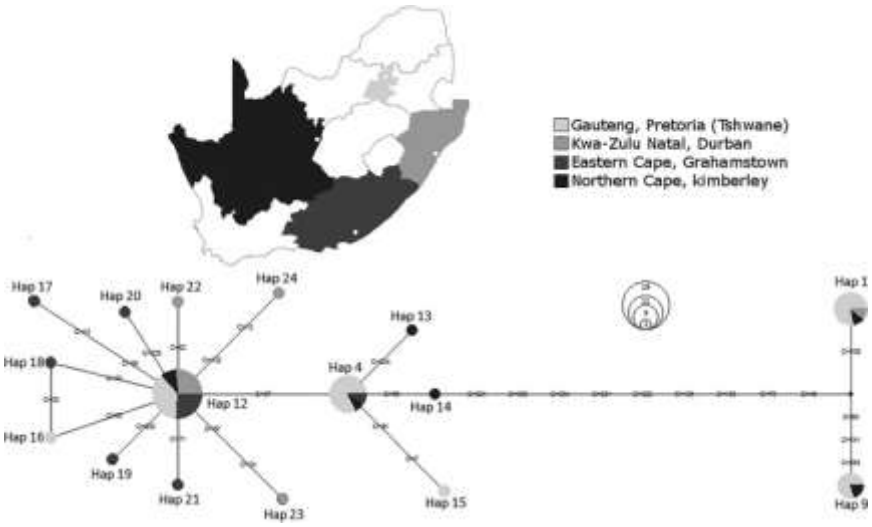

Figure 1: Haplotype diversity of *S. lupi* for cytochrome c oxidase subunit 1. A map of South Africa indicating the nematode origin (white dot) with a haplotype network of the *S. lupi* nematodes from domestic dogs as well as the black backed jackals. The circle sizes are relative to the frequency of the specific haplotypes with the lines between haplotype circles indicating the number of

nucleotide differences between the haplotypes. The pie charts indicate the regions where the haplotypes were derived from.

DNA extraction was performed with the DNeasy® blood and tissue kit (Qiagen) following the protocol for purification of total DNA from animal tissues. Universal helminth primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG) (Bowles et al., 1992) were used to amplify a portion of the cytochrome c oxidase subunit 1 in *S. lupi* (approximately 440 bp). 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. Amplicons were cloned with the StrataClone® PCR cloning kit (Agilent technologies) using StrataClone® SoloPack competent cells (Agilent technologies) according to the manufacturers instructions. Automated sequencing reactions were prepared using BigDye® v3.1 (Applied Biosystems™) chemistry 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.

Sequences were manually edited with CLC Genomics Workbench 8.0.3 (CLC Bio-Qiagen, Aarhus, Denmark). Sequences were compared to those available on the National Center for Biotechnology Information (NCBI) database using the nucleotide basic local alignment search tool (nBLAST) (Altschul et al., 1990). Haplotypes (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 (KJ605487.1 - KJ605493.1) isolated from the red fox, from dogs from Israel (EF394604.1, EF394606.1, EF195133.1, EF394596.1 - EF394598.1), Italy (EF394599.1 - EF394602.1, EF195132.1), Iran (EF394603.1, EF394607.1 - EF394609.1) and Austria (EF394605.1) were obtained from the NCBI database. Only the overlapping region (approximately 156 bp) was used for comparison. *Cylicospirura subaequalis* (GQ342968.1), *Thelazia callipaeda* (NC_018363.1, AB538283.1) and *Gongylonema pulchrum* (AB513725.1) *cox1* sequences were used as outgroups.

Pairwise distance of *cox1* was calculated with MEGA7 (Tamura et al., 2013). Haplotype networks were drawn with the software Network 5 (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 Dendroscope 3 (Huson and Scornavacca, 2012).

Results and discussion

From the 56 nematodes studied, 16 haplotypes of *S. lupi cox1* were identified. Of the 16 haplotypes three have been published previously (HQ674751.1, HQ674754.1, HQ674759.1) (de Waal et al., 2012), the other 13 haplotypes (KY495493-KY495505) are exclusive to this study, with two haplotypes (haplotype 13 and 14) unique to the jackal samples from Kimberley (figure 1). Haplotypes 1, 4 ,9 and 12 are represented in all four regions with 12 being the most common. These shared haplotypes between the jackals and the domestic dogs indicate nematode transmission between the species. This may have conservation implications if the parasite can cross from domestic dogs to other wild carnivores and *vice versa*. This is of concern with respect to the African wild dog (*Lycaon pictus*) since it is currently unknown if *S. lupi* infects these canids.

The Bayesian phylogeny can be divided into three groupings of A, B and C (figure 2). There is strong posterior support for the separation of groups B and C from A with weaker support for the separation of B from C. Similar results can be seen in Al-Sabi's (2014) Neighbor-Joining and Bayesian Inference phylogenetic

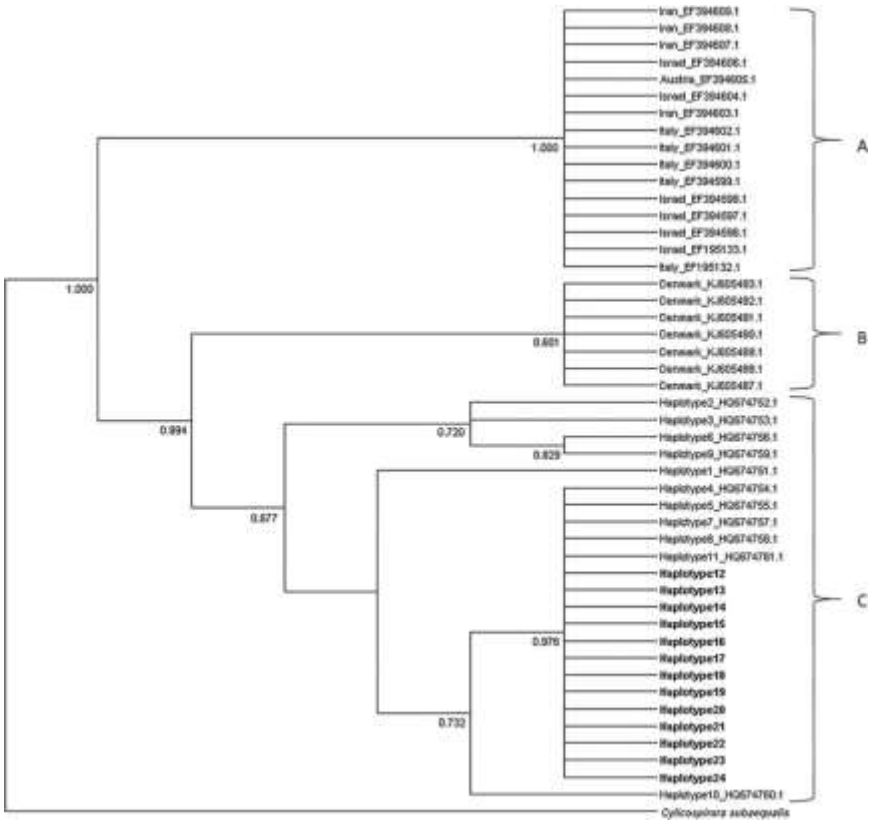

Figure 2: Phylogenetic tree of Bayesian inference of *cox1* sequences from *S. lupi* derived from different continents. A general time-reversible model was used with an inverse gamma substitution rate. Posterior probabilities are based on 1 000 000 generations. The tree was rooted using *Cylicospirura subaequalis*. Three groups are indicated by A, B and C.

trees where the separation between the Denmark and South African samples have slightly lower bootstrap support.

A pairwise differences table was constructed (table 1) to compare the differences between the sequences from the various continents. The pairwise difference between two *Thelazia* isolates is 0.006. When comparing *S. lupi* from domestic dogs from

Table 1
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 *Cylicospirura subaequalis, Gongylonema pulchrum* and 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.

	Hap12	Hap13	Hap14	Hap15	Hap16	Hap17	Hap18	Hap19	Hap20	Hap21	Hap22	Hap23	Hap24	Iran	Israel	Austria	Italy	Denmark	G. Pulchrum	T. callipaeda	T. callipaeda
Haplotype12																					
Haplotype13	0.006																				
Haplotype14	0.013	0.006																			
Haplotype15	0.020	0.013	0.020																		
Haplotype16	0.006	0.013	0.020	0.026																	
Haplotype17	0.013	0.020	0.026	0.033	0.020																
Haplotype18	0.006	0.013	0.020	0.026	0.006	0.020															
Haplotype19	[a]0.000	0.006	0.013	0.020	0.006	0.013	0.006														
Haplotype20	[a]0.000	0.006	0.013	0.020	0.006	0.013	0.006	[a]0.000													
Haplotype21	0.006	0.013	0.020	0.026	0.013	0.020	0.013	0.006	0.006												
Haplotype22	0.006	0.013	0.020	0.026	0.013	0.020	0.013	0.006	0.006	0.013											
Haplotype23	0.013	0.020	0.026	0.033	0.020	0.026	0.020	0.013	0.013	0.020	0.020										
Haplotype24	0.013	0.020	0.026	0.033	0.020	0.026	0.020	0.013	0.013	0.020	0.020	0.026									
Iran_EF394609.1	0.111	0.103	0.103	0.103	0.119	0.111	0.120	0.111	0.111	0.119	0.119	0.128	0.119								
Israel_EF394606.1	0.111	0.103	0.103	0.103	0.119	0.111	0.120	0.111	0.111	0.119	0.119	0.128	0.119	[a]0.000							
Austria_EF394605.1	0.111	0.103	0.103	0.103	0.119	0.111	0.120	0.111	0.111	0.119	0.119	0.128	0.119	[a]0.000	[a]0.000						
Italy_EF394602.1	0.111	0.103	0.103	0.103	0.119	0.111	0.120	0.111	0.111	0.119	0.119	0.128	0.119	[a]0.000	[a]0.000	[a]0.000					
Denmark_KJ605487.1	0.098	0.106	0.098	0.121	0.106	0.098	0.106	0.098	0.098	0.106	0.106	0.114	0.113	0.126	0.126	0.126	####				
G. pulchrum_AB513725.1	0.219	0.209	0.199	0.209	0.230	0.219	0.232	0.219	0.219	0.230	0.230	0.243	0.240	0.172	0.172	0.172	####	0.211			
T. callipaeda_NC_018363.1	0.137	0.145	0.145	0.145	0.145	0.154	0.147	0.137	0.137	0.145	0.145	0.155	0.145	0.125	0.125	0.125	####	0.144	0.230		
T. callipaeda_AB538283.1	0.145	0.154	0.154	0.154	0.154	0.163	0.155	0.145	0.145	0.154	0.154	0.164	0.154	0.133	0.133	0.133	####	0.153	0.230	0.006	
C. subaequalis_GQ342968.1	0.144	0.135	0.135	0.135	0.153	0.144	0.154	0.144	0.144	0.153	0.153	0.163	0.144	0.085	0.085	0.085	####	0.102	0.151	0.179	0.179

[a] Pairwise distance with 2–3 nucleotide differences between sequences are indicated as zero since the difference is too small.

Europe, to the South African *S. lupi* sequences, pairwise differences range from 0.103 to 0.120. The *S. lupi* sequences from red foxes from Denmark have a pairwise distance range from 0.106 to 0.128 when compared to the South African *S. lupi cox1* sequences. When comparing all the *S. lupi* samples to non-related species such as *G. pulchrum*, *T. callipaeda* and *C. subaequalis* the range is between 0.135 and 0.243. 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 genetic differences to European sequences are almost as high as these sequences compared to an outgroup. The same may apply to the South African *Spirocerca* nematodes as there is notable genetic variation between the continents. In addition, the data presented by Al-Sabi and others supports the three groupings that can be seen in the phylogenetic tree in figure 2.

Conclusion

This study only utilized a small region of the *cox1* gene to observe genetic differences in *S. lupi* from different regions and final host species. A larger section of the *cox1* in addition to further genetic analysis would be required to clarify if there is a cryptic species of *S. lupi*. The shared haplotypes between the jackals and the domestic dogs is reason for concern as this species of nematode may spread to other wild canid species such as the endangered African wild dogs. The variation observed within the various hosts

may theoretically produce more virulent parasites (Frank, 1996; Restif, 2009). The higher number of new haplotypes reported compared to a previous study (de Waal et al., 2012) also indicates that there is a high percentage of genetic divergence.

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Conflict of interest

The authors declare no conflicts of interest.

References

Al-Sabi, M.N.S., Hansen, M.S., Chriél, M., Holm, E., Larsen, G., Enemark, H.L., 2014. Genetically distinct isolates of

Spirocerca sp. from a naturally infected red fox (*Vulpes vulpes*) from Denmark. Vet. Parasitol. 205, 389–396.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Aroch, I., Arogeti, I., Marcovics, A., Spiegel, Y., Lavy, E., 2017. In vitro lectin binding to the outer surface of *Spirocerca lupi* at different life-stages. Vet. Parasitol. 235, 94–99.

Aroch, I., Markovics, A., Mazaki-Tovi, M., Kuzi, S., Harrus, S., Yas, E., Baneth, G., Bar-El, M., Bdolah-Abram, T., Segev, G., Lavy, E., 2015. Spirocercosis in dogs in Israel: A retrospective case-control study (2004-2009). Vet. Parasitol. 211, 234–40.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. Annu. Rev. Ecol. Syst. 18, 489–522.

Bailey, W.S., 1972. *Spirocerca lupi*: a continuing inquiry. J. Parasitol. 58, 3–22.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Bowles, J., Blair, D., McManus, D.P., 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54, 165–173.

de Waal, P.J., Gous, A., Clift, S.J., Greeff, J.M., 2012. High within-host genetic variation of the nematode *Spirocerca lupi* in a high-density urban dog population. Vet. Parasitol. 187, 259–266.

Elias, F., Barros, R.M., Santos-Junior, H.L., Eloi, R.S.A., Silva, V., Freitas, F., Fonseca-Alves, C.E., 2016. Pathological alterations in dogs resulting from parasitism by *Spirocerca lupi*. Acta Sci. Vet. 44, 145.

Frank, S.A., 1996. Models of Parasite Virulence. Q. Rev. Biol. 71, 37–78.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Huson, D.H., Scornavacca, C., 2012. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst. Biol. 61, 1061–1067.

Lobetti, R., 2014. Follow-up survey of the prevalence, diagnosis, clinical manifestations and treatment of *Spirocerca lupi* in South Africa. J. S. Afr. Vet. Assoc. 85, 1–7.

Moritz, C., 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. 3, 401–411.

Restif, O., 2009. Evolutionary epidemiology 20 years on: Challenges and prospects. Infect. Genet. Evol. 9, 108–123.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Van der Merwe, L.L., Kirberger, R.M., Clift, S., Williams, M., Kller, N., Naidoo, V., 2008. *Spirocerca lupi* infection in the dog: A review. Vet. J. 176, 294–309.