Comparative mitogenomics of *Spirocerca lupi* from South Africa and China: variation and possible heteroplasmy

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Highlights

- Genetic diversity of 6.1% between mitogenomes of *S. lupi* China and South Africa
- Potential heteroplasmy in *nad2* gene of *S. lupi* South Africa
- High levels of genetic variation in *S. lupi* may be due to high level of heteroplasmy

Abstract

The complete mitochondrial genome of *Spirocerca lupi* isolated from a dog in South Africa was sequenced using next generation sequencing (NGS) technology and the 12 protein coding genes along with the two rRNA genes were compared to 18 other nematode species as well as *S. lupi* from China. The

mitochondrial genome of *S. lupi* South Africa had a mean genetic diversity of 6.1% compared to *S. lupi* China with some variation in nucleotide composition, gene positioning and size. Pairwise distance results indicated slightly higher variation when compared to the pairwise distances of other closely related species, however, this variation was not high enough for it to be considered a cryptic species. Phylogenetic analysis indicated that *S. lupi* from the two continents are very similar. In addition, single nucleotide polymorphisms were detected in the *nad2* gene with ten sequence variants identified from 10 clones from a single nematode, suggesting possible heteroplasmy. The origin of the heteroplasmy is currently unknown but it is speculated to have arisen from accumulated mutations in the mitochondria during somatic replication.

Keywords

Spirocerca lupi, heteroplasmy, parasitic nematode, genetic variation

1 Introduction

Spirocercosis is an infection caused by the parasitic nematode *Spirocerca lupi*. It affects mostly domestic dogs but also infects species such as wolves (Blume et al., 2014), jackals (Bumby et al., 2017; Rothmann and de Waal, 2017), raccoons (Popiołek et al., 2011) and foxes (Ferrantelli et al., 2010; Gomez-Puerta et al., 2018), although these species are infrequently studied. *S. lupi* has a worldwide distribution but is more common in tropical and subtropical regions (Van der Merwe et al., 2008). In South Africa the parasite is endemic (Lobetti, 2014). Endemic infections are continuously present in an area for long periods of time with very limited fluctuation in prevalence (Anderson and

May, 1979). In South Africa the intermediate dung beetles have an incidence of *S. lupi* infection of 13.5% in urban areas and 2.3% in rural areas (Du Toit et al., 2008) and have been reported in domestic as well as wild canids (Rothmann and de Waal, 2017).

Spirocerca lupi causes aortic lesions during its migratory path to the oesophagus. These lesions may rupture if the aortic wall becomes severely damaged (Bailey, 1972). Once in the oesophagus the nematode causes nodules to form that may become necrotic if left untreated (Seibold et al., 1955). It has been proposed that this malignant process is caused by sustained inflammatory response produced due to exposure to excretory and secretory products that are homologous to proteins found in other carcinogenic worms (Porras-Silesky et al., 2021). The parasite is transmitted through intermediate coprophagous beetles as well as paratenic hosts such as lizards, toads, rodents, birds and other small mammals (Bailey, 1972; Ravindran et al., 2014; Van der Merwe et al., 2008; Vashetko and Siddikov, 1999). Once ingested the infective larvae migrate through the coeliac arteries to the thoracic aorta and end up in the oesophagus where their eggs are excreted in the faeces and vomitus (Bailey, 1972; Van der Merwe et al., 2008).

Mitochondrial genomes are circular, double stranded DNA molecules that are widely used in evolutionary studies, due to their maternal inheritance, high rate of sequence divergence and ease of isolation (Harrison, 1989). The first complete mitochondrial genome of *S. lupi* was sequenced in China (Liu et al., 2013). The first mitochondrial genome of *S. lupi* is 13,780 bp in length with 12 protein-coding genes, 22 transfer RNA genes and two ribosomal RNA genes with a A and T rich nucleotide composition of 73.73%. The mitogenome of *S. lupi* should ideally provide datasets for novel molecular markers for studying epidemiology and population genetics. Mitochondrial

genomes have become important in clinical diagnosis as mutations contribute to neurodegeneration as well as other mitochondrial diseases (Kennedy et al., 2012; Tavares and Seuánez, 2017). Complete mitochondrial sequences have been used extensively in phylogenetic and diversity studies of parasitic nematodes (Park et al., 2011; Qiu et al., 2018; Yilmaz et al., 2016).

Using mitochondrial and nuclear ribosomal DNA (rDNA) markers, a new species of *Spirocerca* has recently been described (Rojas et al., 2018), *Spirocerca vulpis*, which is morphologically and genetically different from *S. lupi*. It has been shown that there is high genetic diversity in a partial sequence of the *cox1* gene of *S. lupi* from black backed jackals (*Canis mesomelas*) as well as domestic dogs from four regions in South Africa (Rothmann and de Waal, 2017). A similar study also identified high variability in *cox1* of *S. lupi* from the Andean fox (*Lycalopex culpaeus*) in Peru (Gomez-Puerta et al., 2018). Additionally, another study reported high intra-individual variation in ITS1 of *Spirocerca* spp. (Rojas et al., 2018). This same study also revealed that *S. lupi* clearly divided into two distinct genotypes separated into different geographical areas. These two genotypes combined *S. lupi* from China, India, South Africa and Israel, whereas the second genotype contained only Hungary (Rojas et al., 2018).

The aim of this study was to examine the genetic variation of *S. lupi* between different continents namely Africa (South Africa) and Asia (China) by performing a comparative analysis with all the mitochondrial protein coding genes as well as the mitochondrial ribosomal RNA genes. In addition, the mitochondrial genomes of another 18 nematode species were included in the study for further comparison (Table 1).

Table 1: Nematode mitochondrial assemblies used in this comparative analysis.

Taxon	Accession	Mitogenome size	Reference
Acanthocheilonema viteae	HQ186249.1	13724 bp	(McNulty et al., 2012)
Ascaris suum	NC_001327.1	14284 bp	(Wolstenholme et al., 1994)
Brugia malayi	NC_004298.1	13657 bp	(Ghedin et al., 2007)
Brugia pahangi	AP017680.1	13675 bp	Unpublished
Chandlerella quiscali	NC_014486.1	13757 bp	(McNulty et al., 2012)
Dirofilaria immitis	NC_005305.1	13814 bp	(Hu et al., 2003)
Dirofilaria repens	NC_029975.1	13675 bp	Unpublished
Dracunculus medinensis	NC_016019.1	14628 bp	Unpublished
Gnathostoma doloresi	NC_032073.1	13809 bp	(Sun et al., 2016)
Gongylonema pulchrum	KM264298.1	13798 bp	(Liu et al., 2015)
Heliconema longissimum	NC_016127.1	13610 bp	(Park et al., 2011)
Loa loa	NC_016199.1	13590 bp	(McNulty et al., 2012)
Onchocerca volvulus	AF015193.1	13747 bp	(Keddie et al., 1998)
Philometroides sanguineus	NC_024931.1	14378 bp	(Su et al., 2014)
Setaria digitata	NC_014282.1	13839 bp	(Yatawara et al., 2010)
Spirocerca lupi (South Africa)	MK922357	13804 bp	This publication
Spirocerca lupi (China)	KC305876.1	13780 bp	(Liu et al., 2013a)
Thelazia callipaeda LY1	KY908319.1	13666 bp	(Zhang et al., 2017)
Thelazia callipaeda	NC_018363.1	13668 bp	(Liu et al., 2013b)
Wuchereria bancrofti	JN367461.1	13635 bp	(Ramesh et al., 2012)

2 Materials and methods

2.1 Nematode samples

Nematode samples were obtained from two locations. The first was a dog with a natural spirocercosis infection that was euthanized at the Pyramid Veterinary Clinic in Pretoria (Tshwane Metropole) on 1 December 2017. The second was obtained from Ballito Veterinary Hospital (Ballito, KwaZulu-Natal) on 20 January 2020. The nematode samples were stored in 70% ethanol at 4°C until DNA extraction could be performed.

2.2 DNA extraction

Genomic DNA isolation was performed on a single adult *S. lupi* nematode (obtained from Pyramid Veterinary clinic in Pretoria)

using a method previously described for *C. elegans* (Mello and Fire, 1995), with some modification as described by Min-Ho Lee and Sudhir Nayak (Available online : http://genetics.wustl.edu/tslab/protocols/genomic-stuff/worm-genomic-dna-prep/). The method was scaled down to facilitate genomic DNA isolation from a single *S. lupi* nematode (see supplementary information for a full description of the method).

DNA was extracted from a *S. lupi* adult nematode obtained from Ballito Veterinary Hospital (Ballito, KwaZulu-Natal) using the DNeasy® Blood & Tissue kit (QIAGEN®)

2.3 DNA sequencing

The TruSeq® DNA PCR-Free Library Prep kit (Illumina®) was used to prepare a paired end library by Macrogen Inc. (Seoul, Republic of Korea). The paired end library was sequenced by Macrogen Inc. using the HiSeq® 2500 (Illumina®). The sequences generated for the paired end library were 2 x 100 bp with insert sizes of approximately 350 bp.

2.4 Mitochondrial genome assembly and annotation

The quality of the sequenced reads was evaluated using FastQC v0.11.7 (Andrews, 2010). The mitochondrial DNA was extracted from the genomic data by mapping the reads to the *S. lupi* genome from China (accession number KC305876.1) using CLC bio genomics workbench v.12.0. The consensus sequence was used for annotation and further analysis. Initial annotations were performed on the MITOS web server (Bernt et al., 2013). Final annotations and editing were performed manually with CLC bio genomics workbench. Open reading frames were verified on the Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/) hosted by the

National Centre for Biotechnology Information (NCBI) using the invertebrate mitochondrial genetic code with alternative initiation codons. DNA translations were performed using ExPASy hosted by the Swiss Institute of Bioinformatics Resource Portal (Gasteiger et al., 2003). Basic tRNA structures were created for both *S. lupi* from South Africa and China with the VARNA v.3.93. package (Darty et al., 2009) (Supplementary table 1).

2.5 Pairwise distance and Phylogenetic analysis

Pairwise distance analyses were performed in MEGA 7 (Kumar et al., 2016) with 20 selected nematode mitogenomes (Table 1) downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Sequence alignments were performed using MAFFT v.7. (Katoh and Standley, 2013) with default settings. The first analyses consisted of 12 protein coding genes and two ribosomal DNA sequences from the 20 nematodes. The second with only the 12 protein coding genes. The final analyses consisted of amino acid sequences from the 12 protein coding genes. Bayesian inference analysis was performed with MrBayes v.3.2. (Ronquist et al., 2012) with 10 000 000 generations in two sets, one with 12 protein coding genes, the other with 12 protein coding genes and two ribosomal DNA sequences. The best model predicted with MEGA 7 was the General Time Reversible (GTR) model with Inverse-gamma distributions. Phylogenetic trees were drawn with FigTree v1.4.3 (Rambaut, 2012). Overall mean distance was calculated with MEGA 7 using the maximum composite likelihood method.

2.6 Long PCR cloning and *nad2* sequencing

A long PCR was performed with DNA obtained from the second nematode (Ballito, KwaZulu-Natal) with the designed forward primer W2F (5’ TCATTTGGGGTTGTTGGTT) and reverse

primer JB8 (5’ CAGACATGAATACCCAGAGAAAT). These primers were designed with Primer BLAST hosted by the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to cover the entire *cox1* and *nad2* region of *S. lupi*, a section of approximately 4200 bp. The long PCR was performed with the GoTaq® Long PCR Master Mix (Promega) using the standard PCR protocol. The amplicon was resolved on a 1 x TAE agarose gel stained with crystal violet (Rand, 1996) according to the S.N.A.P.™ UV-Free Gel Purification Kit protocol (Invitrogen). The PCR product was excised from the crystal violet gel and cleaned with High Pure PCR Product Purification Kit (Roche). The amplicon was cloned with pGEM®-T Easy Vector Systems (Promega). The ligation was prepared with a 1:10 vector to insert ratio with a ligation period of two weeks at 4°C. DH5α Competent Cells (Thermo Scientific) were used for transformation. The cells were grown on LB plates supplemented with 100 µg/ml Ampicillin, 0.5mM IPTG and 80µg/ml X-Gal. Several colonies were selected for plasmid extraction with the Zyppy™ Plasmid Miniprep Kit (Zymo Research). Only the *nad2* region of ten plasmids were sequenced using two forward primers, W2F (5’ TCATTTGGGGTTGTTGGTT) and SLN2F (5' GTAGTTATTGTTTCTACGGAGTC) as well as two reverse primers, W2R (5’ ACACCAAAATTATACCCCG) and SLN2R (5’ CGTCACAATCCTCTAATTACAAATC). These primers were designed with Primer BLAST as previously stated. The sequencing was performed by the ACGT sequencing facility at the University of Pretoria with Big Dye v3.1 sequencing technology on the ABI 3500. Sequences were aligned and edited on the CLC bio genomics workbench v.12.0.

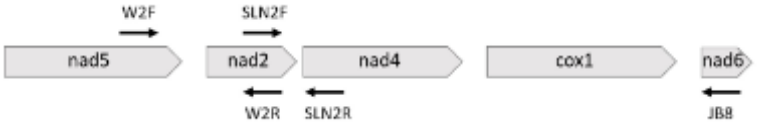

Fig. 1. Segment of the mitochondrial genome showing the annealing position of the long PCR primers used. Primer positions are approximate.

3 Results

Approximately 17 µg of DNA was recovered from the first nematode with an absorbance ratio of 1.8 at 260nm/280nm. Sequencing data received from Macrogen consisted of approximately 112 million reads of 100bp with a Phred score of 30 or above. No quality editing was required. The final mitochondrial assembly consisted of 13 804 bp with a CG content of 25.6%. The mitochondrial genome of *S. lupi* South Africa (accession number MK922357) consists of 12 protein coding genes, 2 ribosomal RNA genes and 22 tRNAs, which is consistent with *S. lupi* from China (Table 2) as well as other nematode species. Basic tRNA structures were very similar between the two *S. lupi* mitochondrial genomes with only single nucleotide variations (Supplementary table 1). All the anticodons were the same as previously reported for *S. lupi* from China (Liu et al., 2013).

Pairwise distance results from the 12 protein coding genes as well as the two rRNA genes indicate variation of 0.062 between *S. lupi* from South Africa and *S. lupi* from China, which is almost four times higher than the variation between two *Thelazia callipaeda* specimens at 0.017 (Table 3). In comparison it is lower than the variation between closely related species *Brugia malayi* and *Brugia pahangi* at 0.108. The pairwise distances for only the 12 protein coding genes yielded similar results (Supplementary table 2). Pairwise distance results from the amino acid sequences of the 12 protein coding genes indicate variation of 0.087 between *S. lupi* South Africa and *S. lupi* China

Table 2: Comparison between *Spirocerca lupi* mitochondrial genome from South Africa and China

	S. lupi South Africa					*S. lupi* China				
Gene	Position	Length	Ini/Ter	Anticodon	AA	Position	Length	Ini/Ter	Anticodon	AA
cox1	1-1656	1656	ATG/TAA		551	1-1650	1650	ATG/TAA		549
trnW(tga)-Trp	1663-1717	55		TCA		1657-1714	58		TCA	
nad6	1757-2215	459	TTG/TAA		152	1751-2209	459	TTG/TAA		152
trnR(cgt)-Arg	2214-2269	56		ACG		2207-2266	60		ACG	
trnQ(caa)-Gln	2269-2322	54		TTG		2263-2316	54		TTG	
Cytb	2335-3388	1053	ATT/TAA		358	2315-3397	1083	ATT/TAA		360
trnL1(cta)-Leu	3410-3463	54		TAG		3396-3450	55		TAG	
cox3	3461-4243	783	ATA/TAA		260	3448-4230	783	ATA/TAA		260
Non-coding region	4244-4638	395				4231-4630	400			
trnD(gca)-Ala	4639-4696	58		TGC		4631-4692	62		TGC	
trnL2(tta)-Leu	4701-4754	54		TAA		4689-4742	54		TAA	
trnN(aac)-Asn	4759-4816	58		GTT		4747-4804	58		GTT	
trnM(atg)-Met	4819-4875	57		CAT		4807-4864	58		CAT	
trnK(aaa)-Lys	4879-4935	57		TTT		4867-4924	58		TTT	
nad4L	4943-5170	228	ATG/TAG		75	4932-5159	228	ATG/TAG		75
rrnS	5181-5868	688				5170-5855	686			
trnY(tac)-Tyr	5868-5923	56		GTA		5855-5910	56		GTA	
nad1	5921-6799	879	TTG/TAA		292	5908-6816	909	TTG/TAA		302
trnF(ttc)-Phe	6800-6855	56		TTG		6785-6843	59		TTG	
atp6	6862-7446	585	ATT/TAA		194	6847-7431	585	ATT/TAG		194
trnI(atc)-Ile	7450-7506	57		GAT		7435-7491	57		GAT	
trnG(gga)-Gly	7507-7562	56		TCC		7492-7546	55		TCC	
cox2	7565-8252	688	ATG/TAA		228	7549-8253	705	ATG/TAG		234
trnH(cac)-His	8259-8315	57		GTG		8244-8302	59		GTG	
rrnL	8314-9300	987				8301-9288	988			
nad3	9293-9628	336	ATT/TAA		111	9281-9616	336	TTG/TAA		111
trnC(tgc)-Cys	9628-9682	55		GCA		9616-9670	55		GCA	
trnS2(tca)-Ser	9683-9734	52		TGA		9673-9726	54		TGA	
trnP(cct)-Pro	9738-9790	53		AGG		9730-9787	58		AGG	
trnD(gac)-Asp	9858-9912	54		GTC		9847-9900	54		GTC	
trnV(gta)-Val	9914-9967	54		TAC		9902-9955	54		TAC	
nad5	9971-11561	1591	TTG/TAA		529	9959-11551	1593	TTG/TAG		530
trnE(gaa)-Glu	11562-11618	57		TTC		11550-11606	57		TTC	
trnS1(aga)-Ser	11619-11668	50		TCT		11607-11656	50		TCT	
nad2	11672-12514	843	TTG/TAG		280	11637-12485	849	ATG/TAG		282
trnT(aca)-Thr	12516-12573	58		TGT		12487-12543	57		TGT	
nad4	12598-13803	1206	TTG/TAG		401	12544-13773	1230	TTG/TAG		409

Table 3: Pairwise distance generated in MEGA with 12 protein coding genes and two ribosomal RNAs of 20 nematode species (top half) and the pairwise distance of amino acid sequences of 12 protein coding genes of 20 nematode species (bottom half).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*A. Virteae*		1.006	0.239	0.249	0.263	0.224	0.204	0.982	1.180	0.375	0.568	0.211	0.206	1.389	0.233	0.332	0.367	0.398	0.399	0.227
2	*A. suum*	1.303		0.997	1.027	0.991	0.953	0.910	0.674	0.496	1.033	1.049	0.941	0.964	0.938	0.938	0.991	1.050	1.046	1.051	0.938
3	*B. malayi*	0.260	1.277		**0.108**	0.254	0.242	0.210	1.014	1.221	0.385	0.561	0.201	0.218	1.436	0.239	0.343	0.379	0.407	0.411	0.134
4	*B. pahangi*	0.260	1.272	**0.053**		0.264	0.245	0.222	1.042	1.222	0.386	0.584	0.207	0.225	1.447	0.244	0.350	0.386	0.411	0.414	0.140
5	*C. quiscali*	0.309	1.307	0.287	0.282		0.259	0.223	0.967	1.171	0.387	0.540	0.222	0.234	1.324	0.249	0.346	0.383	0.412	0.416	0.241
6	*D. immitis*	0.280	1.288	0.270	0.266	0.313		0.141	0.981	1.163	0.358	0.560	0.204	0.177	1.389	0.216	0.316	0.349	0.395	0.399	0.219
7	*D. repens*	0.255	1.254	0.235	0.234	0.280	0.160		0.909	1.149	0.317	0.510	0.177	0.153	1.347	0.183	0.291	0.319	0.375	0.375	0.188
8	*D. medinensis*	1.345	0.797	1.374	1.355	1.398	1.372	1.337		0.815	1.014	1.073	0.938	0.963	0.653	0.918	**1.004**	1.066	1.042	1.042	0.952
9	*G. doloresi*	1.335	0.430	1.341	1.330	1.331	1.333	1.314	0.809		1.203	1.222	1.188	1.158	1.007	1.149	1.148	1.194	1.204	1.199	1.172
10	*G. pulchrum*	0.465	1.303	0.444	0.433	0.495	0.453	0.427	1.410	1.330		0.562	0.336	0.348	1.396	0.333	0.325	0.357	0.436	0.442	0.362
11	*H. longissimum*	0.734	1.296	0.713	0.711	0.734	0.728	0.684	1.429	1.339	0.750		0.531	0.557	1.371	0.528	0.552	0.590	0.582	0.585	0.550
12	*L. loa*	0.248	1.279	0.212	0.208	0.278	0.252	0.211	1.382	1.374	0.429	0.681		0.161	1.343	0.203	0.302	0.338	0.383	0.387	0.181
13	*O. volvulus*	0.232	1.256	0.209	0.203	0.276	0.204	0.171	1.324	1.347	0.424	0.669	0.149		1.406	0.201	0.299	0.331	0.386	0.389	0.194
14	*P. sanguineus*	1.532	0.915	1.546	1.512	1.562	1.510	1.519	0.654	0.935	1.532	1.592	1.528	1.508		1.336	1.365	1.435	1.398	1.396	1.362
15	*S. digitata*	0.298	1.285	0.275	0.266	0.325	0.278	0.245	1.313	1.355	0.443	0.728	0.268	0.245	1.508		0.295	0.328	0.374	0.377	0.219
16	*S. lupi SA*	0.421	1.236	0.405	0.400	0.447	0.409	0.390	1.355	1.261	0.384	0.737	0.406	0.380	1.490	0.397		**0.062**	0.395	0.397	0.320
17	*S. lupi CH*	0.503	1.337	0.487	0.485	0.523	0.481	0.454	1.475	1.356	0.458	0.807	0.487	0.450	1.611	0.463	**0.087**		0.425	0.429	0.349
18	*T. callipaeda LY1*	0.521	1.384	0.492	0.488	0.541	0.500	0.491	1.406	1.452	0.564	0.833	0.504	0.485	1.548	0.489	0.520	0.583		**0.017**	0.393
19	*T. callipaeda*	0.517	1.368	0.492	0.489	0.536	0.498	0.486	1.392	1.429	0.564	0.826	0.499	0.480	1.526	0.486	0.522	0.584	**0.024**		0.394
20	*W. bancrofti*	0.261	1.240	0.098	0.089	0.286	0.260	0.224	1.347	1.292	0.430	0.707	0.203	0.208	1.508	0.277	0.391	0.478	0.481	0.481	

which is higher than the 0.024 recorded between the two *T. callipaeda* specimens (Table 3). This value is also slightly higher than the recorded value between the two *Brugia* species at 0.053.

The Bayesian analysis indicated close relatedness between *S. lupi* from China and South Africa for the protein coding genes and rRNA phylogenetic tree (Figure 2) as well as the protein coding genes phylogenetic tree (no rRNA data included) (Supplementary figure 1). Transition/transversion mutations observed by comparing *S. lupi* South Africa to China indicate a ratio of 2:1 with the only bias between substitutions of G and T. The rest of the substitutions appear fairly equal (Figure 3).

Sequencing of the *nad2* region of *S. lupi* revealed single nucleotide polymorphisms which suggests possible heteroplasmy. Of the ten cloned sequences, the same number of variants were identified if alignment gaps were considered. The ten *nad2* variants are shown in supplementary figure 2. The potential heteroplasmic single nucleotide polymorphisms and indels are listed in Tables 4 and 5.

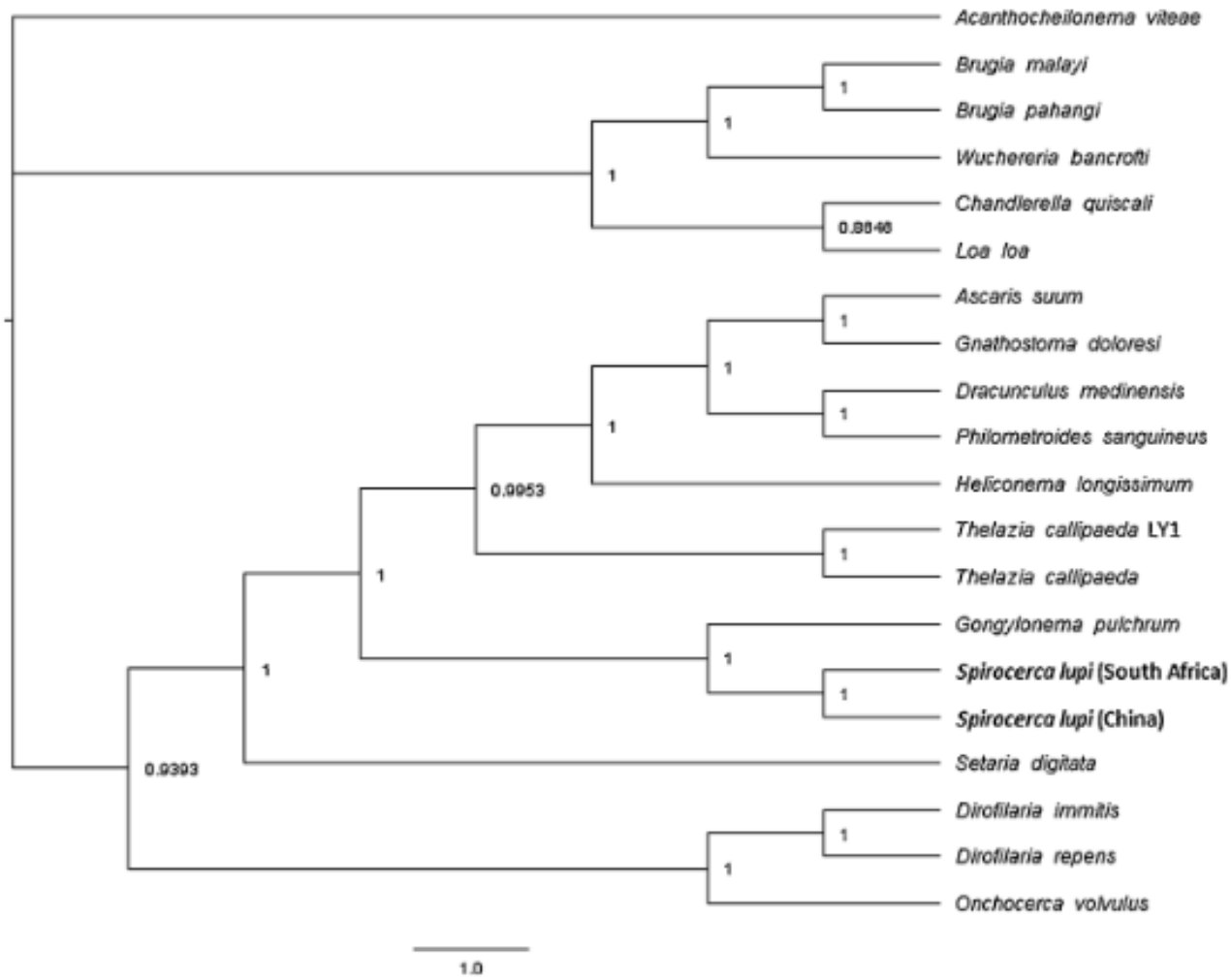

Fig. 2. Bayesian phylogenetic tree of 20 nematode species based on 12 mitochondrial protein coding genes and two rRNA genes. The number above the branches represents the Bayesian posterior probabilities.

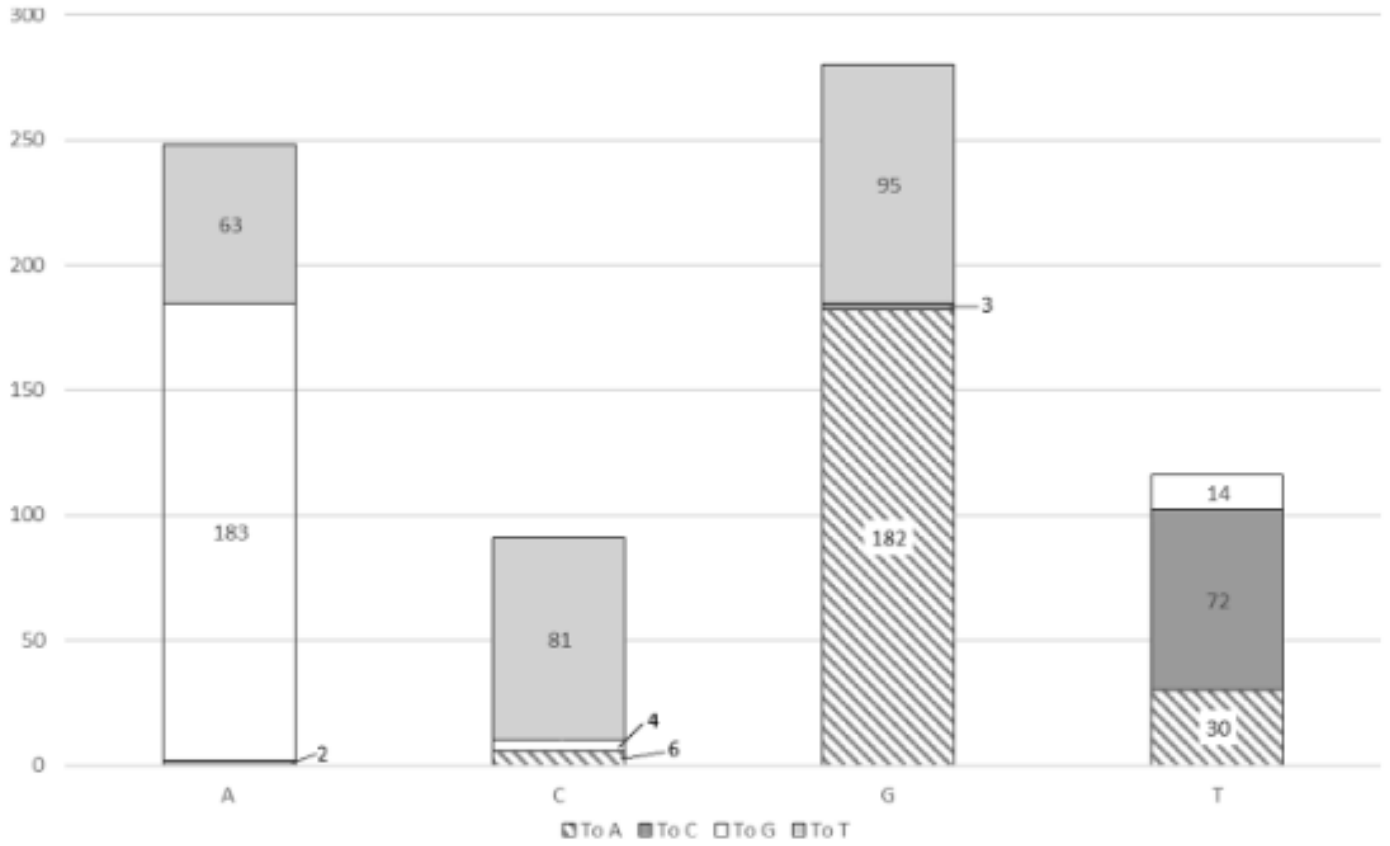

Fig. 3. Transition/transversion mutations observed by comparing *S. lupi* South Africa to China.

Table 4: *S. lupi* partial *nad2* single nucleotide polymorphisms found in ten variants from 10 clones from a single nematode.

Gene	Position	Ref Allele	Var allele	Variant	Var freq	Type	Ref Codon	Ref AA	Var Codon	Var AA	Type
nad2	50	T	C	1	10 %	Transition	GTG	Val	GCG	Ala	Non-syn
	142	T	C	3	10 %	Transition	TTT	Phe	CTT	Leu	Non-syn
	280	T	C	10	10 %	Transition	TGA	Trp	CGA	Arg	Non-syn
	747	T	G	4	10 %	Transversion	GGT	Gly	GGG	Gly	Syn
	752	G	T	5	10 %	Transversion	TGG	Trp	TTG	Leu	Non-syn
	774	G	A	7	10 %	Transition	ACG	Thr	ACA	Thr	Syn
	796	T	C	1	10 %	Transition	TAT	Tyr	CAT	His	Non-syn
	797	A	T	1,6,8,9	40%	Transversion	TAT	Tyr	TTT	Phe	Non-syn
	799-800	TA	CT	1	10 %	Both	TAT	Tyr	CTA	Leu	Non-syn
	824	T	C	10	10 %	Transition	ATG	Met	ACG	Thr	Non-syn

Table 5: *S. lupi* partial *nad2* indels found in ten variants from 10 clones from a single nematode.

Gene	Position	Ref Allele	Var allele	Variant	Var freq	Type	Ref Codon	Ref AA	Var Codon	Var AA	Type
nad2	20	T	_	1	10 %	Deletion	TTG	Leu	TGT	Cys	Frameshift
	21	_	_	3,5	20 %	Insertion	TTG	Leu	TTT	Phe	Frameshift
	117	T	_	3,10	20 %	Deletion	TTT	Phe	TTA	Leu	Frameshift
	375	T	_	2,10	20 %	Deletion	TTT	Phe	TTG	Leu	Frameshift
	374-375	TT	_ _	3	10 %	Deletion	TTT	Phe	TGT	Cys	Frameshift
	520	T	_	4,5	20 %	Deletion	TGT	Cys	GTG	Val	Frameshift
	590	_	T	3	10 %	Insertion	TTG	Leu	TTT	Phe	Frameshift
	618	_	T	3	10 %	Insertion	TTA	Leu	TTT	Phe	Frameshift
	618-620	_ _ _	TTA	5	10 %	Insertion	TTA	Leu	TTT, TAA	Phe, Stop	Nonsense
	636	G	_	10	10 %	Deletion	TGT	Cys	TTT	Phe	Frameshift
	650-651	_ _	TT	2	10 %	Insertion	TTT	Phe	TTG	Leu	Neutral
	670	_	T	5	10 %	Insertion	GGT	Gly	TGG	Trp	Frameshift
	699	T	_	7	10 %	Deletion	TTT	Phe	TTA	Leu	Frameshift
	710	_	T	9	10 %	Insertion	TGT	Cys	TTG	Leu	Frameshift
	801	T	_	1	10 %	Deletion	TAT	Tyr	TAG	Stop	Nonsense
	802	G	_	1,6	20 %	Deletion	GAT	ASP	ATT	Ile	Frameshift
	817-819	T	_ _ _	5	10 %	Deletion	TTG	Leu	TTT	Phe	Frameshift
	818	T	_	9	10 %	Deletion	TTG	Leu	TGT	Cys	Frameshift
	819	_	T	3,4	20 %	Insertion	TTG	Leu	TTT	Phe	Frameshift

4 Discussion

Genetic diversity in parasitic nematodes that are separated by vast geographic distances may affect the efficacy of treatment regimes (Hawash et al., 2015). In *S. lupi* there are 737 polymorphic sites between South Africa and China with 94% BLAST identity on NCBI, and an overall mean genetic distance of 6.1%. Pairwise distances indicate that there is some variation between *S. lupi* China and South Africa. Although *S. lupi* South Africa and China are still very similar, we speculate that the discrepancy in the pairwise variation between the 12 protein coding genes and the two rRNA genes of *S. lupi* South Africa and *S. lupi* China compared to the same genes of the two *T. callipaeda* specimens (LY1 from Luoyang, Henan province, China and *T. callipaeda* from Zhanjiang, Guangdong Province, China) is due to geographical isolation. Both the *T. callipaeda* specimens were isolated in China whereas the *S. lupi* samples were from two different continents. Genetic variation between continents due to geographical separation could lead to genetic differentiation (Huyse et al., 2005). *Trichuris* species infecting humans and pigs were found to be phylogenetically distinct. Across geographical regions, genetic and protein distances of human *Trichuris* were 19% and 15%, respectively (Hawash et al., 2015). This is similar to a study where genetic differences between sibling nematode species differed between 10% to 20% (Blouin, 2002).

There were slight differences in gene positioning and sizes of *S. lupi* from China and South Africa, especially for *cytochrome b*, *nad1*, *cox2* and *nad4* (Table 2). *Cytochrome b*, *cox2* and *nad5* did not have distinct stop codons. These genes will require the addition of a 3' A residue to the mature mRNA through polyadenylation in order to complete the UAA stop codon (Jacob et al., 2009). This is similar to that of *Gongylonema pulchrum*

mitochondrial *nad1*, *atp6*, *cox2*, *nad3* and *nad5* (NC_026687.1) (Liu et al., 2015).

A strong mutational bias of G/C to A/T has been reported in *C. elegans* (Konrad et al., 2017). We found that comparing the mitochondrial genome of *S. lupi* from South Africa to that of China that the same transitional mitochondrial mutation bias is present (Supplementary figure 2). This mutational bias is common in nematode genomes, creating pressure towards higher AT content of genomes (Blouin et al., 1998; Denver et al., 2012; Weller et al., 2014).

No sequencing reads were obtained for a section of the *nad2* gene during HiSeq® 2500 (Illumina®) sequencing which resulted in a gap of approximately 200 bp in the mitogenome assembly. Primers were designed to amplify, clone and sequence the missing section. The final sequences revealed extensive single nucleotide polymorphisms. In order to eliminate potential pseudogene amplification and to determine if the polymorphisms exist in other *S. lupi* nematodes, a nematode from a different region (Ballito, KwaZulu-Natal) was used to amplify and sequence ten clones of *nad2*. From these ten clones ten variants were identified suggesting the presence of possible heteroplasmy (Supplementary figure 2).

The ten sequences were all variable if alignment gaps were considered. If alignment gaps were not considered, sequences 1-7 and 10 were variable with sequences 8 and 9 the same as 6. Only sequence 1, 6, 8 and 9 had long open reading frames. The other sequences had shorter (<750 bp) open reading frames indicating that they may be non-functional. Sequences 1 and 8 are available online (Accession MW669976 and MW669977).

Mitochondrial genomes are widely considered as effective targets for genetic markers for diversity and population phylogenetic studies (Palevich et al., 2019; Shi et al., 2018; Xie

et al., 2019). Heteroplasmy in the mitochondrial genomes of animals range from phenotypically neutral to the bipartite mitochondrial genome of *Ruizia karukerae* (Kim et al., 2018). Heteroplasmy has been reported in several species such as *Drosophila* (Solignac et al., 1983), mice (Gyllensten et al., 1991) and the nematode *Caenorhabditis elegans* (Liau et al., 2007). Heteroplasmy can be detrimental to nematodes by reducing fitness (Liau et al., 2007) but stable heteroplasmy is also likely as has been shown in *C. elegans* and *Drosophila* (Tsang and Lemire, 2002; Volz-Lingenhöhl et al., 1992). Heteroplasmy may arise through several mechanisms of mutation that are still understudied (Wernick et al., 2016). Mitochondrial genomes can also be inherited from fathers as well as mothers (Luo et al., 2018), as has been demonstrated in plants such as the cucumber where pollen transmits paternal mtDNA (Havey, 1997). In *C. elegans* the sperm's mitochondria are destroyed through autophagy (Al Rawi et al., 2011; Sato and Sato, 2011), as well as in mice (Rojansky et al., 2016). This would therefore not be the expected cause of heteroplasmy in *S. lupi*. We speculate the cause of some heteroplasmy to be due to mutations which have accumulated during mitochondrial replication in somatic cells (Ladoukakis and Zouros, 2017). In addition, some heteroplasmy may have been inherited from the mother. This is possibly due to a mechanism known as Muller's Ratchet, where non-recombining genomes accumulate mutations much faster than recombining genomes. Mitochondrial genomes of animals are susceptible to Muller's Ratchet which could affect the rate of mutation (Howe and Denver, 2008). Heteroplasmy should be considered in future phylogenetic studies as high variation could potentially be attributed to the various heteroplasmies.

5 Conclusion

Some genetic variation between *S. lupi* from South Africa compared to China is present possibly due to geographic separation. Regardless, *S. lupi* from China and South Africa are still closely related and fall under the same genotype as reported by Rojas *et al.* (2018a). High variation in *S. lupi* may also be due to a high level of heteroplasmy in the mitogenome. This study is the first known publication presenting evidence of heteroplasmy in *S. lupi nad2*. The mechanism of heteroplasmy is proposed to be an accumulation of mutations in the mtDNA due to somatic replication. The extent of heteroplasmy throughout the *S. lupi* mitogenome is unknown, since only a single gene was examined for this study.

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Conflict of interest

The authors declare no conflict of interest.

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