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Characterisation of the mitochondrial genome of *Parafilaroides normani* (lungworm) of *Arctocephalus pusillus doriferus* (Australian Fur Seal)

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Abstract *Parafilaroides normani* is a metastrongyloid nematode of clinical relevance in some species of pinnipeds. The taxonomy, biology and epidemiology of this and related species are not well understood. Mitochondrial (mt) DNA can provide markers for studies in these areas, but genetic data are scant. Here, we characterised the mt genome of *P. normani*. Adults of this nematode were collected from *Arctocephalus pusillus doriferus* from Phillip Island, Victoria, Australia, and genomic DNA was extracted from individuals. The complete mt genome was amplified from DNA from a single, adult male by long-range PCR, as two overlapping amplicons (~5 kb and ~10 kb), and then sequenced employing 454 technology (Roche). The mt genome was assembled from reads of ~300 bp and annotated using an established bioinformatic pipeline. This circular mt genome is 13,411 bp and contains two ribosomal RNA, 12 protein-coding and 22 transfer RNA genes, consistent with those of other lungworms studied to date. This mt genome is intended to provide a source of genetic markers for future investigations of the biology, epidemiology and systematics of *Parafilaroides* spp. and related lungworms.

Keywords Pinnipeds • *Parafilaroides normani* • pinnipeds • lungworm • mitochondrial genome

Introduction

Parafilaroides Dougherty 1946 (Metastrongyloidea: Filaroididae) is a genus of delicate, small lungworms of pinniped marine mammals. To date, seven valid species of *Parafilaroides* are recognised, namely *P. gymnurus* Railliet 1899; *P. decorus* Dougherty and Herman 1947; *P. hydrurgae* Mawson 1953; *P. hispidus* Kennedy 1986; *P. measuresae* Dailey 2006; *P. gullandae* Dailey 2006; and *P. normani* Dailey 2009 (cf. Dailey 2006, 2009). *P. gymnurus*, *P. hydrurgae*, *P. hispidus*, *P. measuresae* and *P. gullandae* are found in Earless or True seals (Phocidae). *P. decorus* infects the Steller (*Eumetopias jubatus*) and Californian (*Zalophus californianus*) sealions (Dailey 2006), and the most recently described species, *P. normani*, is known to infect Eared seals, including *Arctocephalus pusillus doriferus*, *A. forsteri* and *A. tropicalis* (Otariidae) (Dailey 2009)

Parafilaroides spp. are known to cause respiratory disease in pinnipeds, which can result in high mortality rates (Dailey 1970; Onderka 1989; Siebert et al. 2007). Although there have been some studies of potential intermediate hosts of these parasites (Dailey 1970; Bergeron et al. 1997a,b; Houde et al. 2003), life cycles, transmission and pathogenesis of the disease are largely unknown. Molecular techniques using suitable genetic markers could readily underpin such fundamental studies. In addition to nuclear ribosomal DNA regions (cf. Carreno and Nadler 2003; Johnson et al. 2004; Chilton et al. 2006; Lehnert et al. 2010), mitochondrial (mt) genomes can provide markers suitable for systematic and epidemiological studies of nematodes, with a focus on, for example, exploring transmission patterns and discovering population variants or cryptic species (Hu and Gasser 2006; Jex et al. 2010a). Here, we characterised the mt genome of *P. normani* using an established massively parallel sequencing-bioinformatic platform and discuss the potential of this genome as a source of genetic markers for such future investigations.

Materials and methods

Adults of *P. normani* were collected by Dr Richard Norman from the lung of a (frozen) dead specimen of fur seal (*Arctocephalus pusillus doriferus*) from Phillip Island, Victoria, Australia, washed extensively in physiological saline and then stored at -80 °C. Genomic DNA was then isolated from a single adult male specimen using a small-scale sodium dodecyl-sulphate (SDS)/proteinase K digestion and column-purification (Wizard DNA Clean-Up kit, Promega, USA) (cf. Gasser et al. 2006). The identity of this specimen was verified by PCR-based sequencing of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA using an established method (Gasser et al. 2006). Subsequently, the complete mt genome was amplified from the genomic DNA from the single male worm by long-range PCR (BD Advantage 2, BD Biosciences) as two overlapping amplicons (~5 kb and ~10 kb) (cf. Hu et al. 2007). PCR products were treated with shrimp alkaline phosphatase and exonuclease I (Werle et al. 1994), and DNA quantified spectrophotometrically. Following agarose electrophoretic analysis, both amplicons (2.5 µg of each) were pooled and sequenced using the 454 Genome Sequencer FLX (Roche) (Margulies et al. 2005). The mt genome (GenBank accession no. ---to be entered---) was assembled from reads of ~300 bp using the program CAP3 (Huang and Madan 1999) and then annotated using an established bioinformatic pipeline (Jex et al. 2010b).

93   **Results**

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95   The circular mt genome sequence of *P. normani* is 13,411 bp in length (Fig. 1). It contains
96   two ribosomal genes, 12 protein-coding (*cox1-3*, *nad1-6*, *nad4L*, *atp6* and *cytB*) and 22
97   tRNA genes. The gene arrangement in the mt genome of *P. normani* was consistent with
98   GA2 representing all other nematodes of the order Strongylida studied to date (see Hu and
99   Gasser 2006; Jex et al. 2010b). All of the 36 genes are transcribed in the same direction
100   (5'→3') (Fig. 1). Overall, the genome is AT-rich, as expected for strongylid nematodes (Hu
101   and Gasser, 2006), with T being the most favoured nucleotide and C the least favoured. The
102   nucleotide contents were 21.0% (A), 6.5% (C), 21.3% (G) and 51.2% (T) (Table 1). The
103   longest non-coding (AT-rich) region, located between the *nad5* and *trnA*, was 126 bp in
104   length (see Fig. 1); its AT-content was 81.0%, significantly greater than for all other parts of
105   the mt genome (Table 2).

106   The *rrnS* and *rrnL* genes were identified by sequence comparison with the mt genome of
107   *Metastrongylus salmi*. The *rrnS* gene was located between *trnE* and *trnS* (UCN), and *rrnL*
108   was between *trnH* and *nad3*. The two genes were separated from one another by the protein
109   genes *nad3*, *nad5*, *nad6* and *nad4L* (Fig. 1). The size of the *rrnS* gene of *P. normani* was 696
110   bp. The size of the *rrnL* gene of *P. normani* was 961 bp. The lengths of these two genes were
111   similar to those of other Metastrongyloidea for which mt genomes are known (696-699 bp
112   for *rrnS*, and 958-961 bp for *rrnL*; cf. Jex et al. 2010b; Gasser et al. 2012a; Lv et al. 2012;
113   Jabbar et al. 2013a), and amongst the shortest for metazoan organisms (cf. Lavrov and
114   Brown 2001).

115   The prediction of initiation and termination codons for the protein-coding genes of *P.*
116   *normani* (Table 2) showed that the commonest start codon was ATT (for six of 12 proteins),
117   followed by ATG (three genes), TTG (two genes) and ATA (one gene). All mt protein genes
118   were predicted to have a TAA or TAG translation termination codon. The preferred

nucleotide usage at the third codon position of mt protein genes of *P. normani* reflects the overall nucleotide composition of the mt genome. At this position, T is the most frequently, and C the least frequently used. The codons ending in G have higher frequencies than the codons ending in A, which, interestingly, is similar to, for example, *Aelurostrongylus abstrusus* and *Angiostrongylus vasorum* (see Gasser et al. 2012a; Jabbar et al. 2013a), but distinct from some other members of the Strongylida, including *Ancylostoma duodenale*, *Caenorhabditis elegans*, *Necator americanus* and *Strongyloides stercoralis* (see Okimoto et al. 1992; Hu et al. 2002, 2003). As the usage of synonymous codons appears to be preferred in gene regions of functional importance, codon bias appears to be linked to selection at silent sites and maximize translation efficiency (Sharp and Matassi 1994; Durent and Mouchiroud 1999).

The AT bias in the genome is also reflected as a bias in the amino acid composition of predicted proteins. The AT-rich codons represent the amino acids Phe, Ile, Met, Tyr, Asn and Lys, and the GC-rich codons represent Pro, Ala, Arg and Gly. In the mt genome of *P. normani*, the most frequently used codons were TTT (Phe), TTA (Leu), ATT (Ile), TTG (Leu), TAT (Tyr), GGT (Gly) and GTT (Val). Five of these codons are AT-rich, and one of them is GC-rich. Six of the seven codons contained an A or a T in the two positions, except for GGT (Gly), which contained a T only in the third position. None of them had a C at any position. The least frequently used codons were CTA, CTG (Leu), ATC (Ile), GTC (Val), AGC (Ser), CCC (Pro), GCC (Ala), TAC (Tyr), CAC (His), AAC (Asn), CGA (Arg), TCC (Ser) and GGC (Gly). All four GC-rich codons were represented here, and every codon had at least one C. When the frequencies of synonymous codons within the AT-rich group were compared, such as Phe (TTT, 16.0%; TTC, 0.3%), Ile (ATT, 5.2%; ATC, 0.1%), Tyr (TAT, 5.1%; TAC, 0.1%) and Asn (AAT, 2.5%; AAC, 0.1%), the frequency was always less if the third position was a C.

Twenty-two tRNA gene sequences were identified in the mt genome of *P. normani*. These sequences ranged from 53-60 nt in length. The tRNA structures had a 7 bp amino-acyl arm, a 4 bp DHU arm, a 5 bp anticodon stem, a 7 base anticodon loop, a T always preceding an anticodon as well as a purine always following an anticodon. Twenty of the 22 tRNA genes (i.e. excluding the two *trnS* genes) have a predicted secondary structure with a 4 bp DHU stem and a DHU loop of 5-10 bases, in which the variable T ψ C arm and loop are replaced by a “TV-replacement loop” of 4-11 bases, in accordance with most nematodes whose mt genomes are known (Hu and Gasser 2006). The mt *trnS* for *P. normani* has a secondary structure consisting of a DHU replacement loop of 7 bases, 3 bp T ψ C arm, T ψ C loop of 4-8 bases and a variable loop of 3 bases, consistent with many other nematodes (e.g., Okimoto et al. 1992; Keddie et al. 1998; Jex et al. 2010a; Park et al. 2011), but distinct from *T. spiralis* and *T. murrelli* (Enoplea) (see Lavrov and Brown 2001; Webb and Rosenthal 2010, 2011).

Discussion

The mt genome of *P. normani* described here is intended to provide a resource for future molecular taxonomic, epidemiological or ecological studies of this species, and provides a basis for a future comparative studies of mt genomes of all seven recognised species of *Parafilaroides*. Guided by sliding window analysis (cf. Mohandas et al. 2013; Jabbar et al. 2013b) among the mt genomes of recognised species of *Parastrongyloides*, oligonucleotide primers could be designed to conserved regions flanking “variable sequence tracts” in the mt genome considered as informative for specific and sub-specific identification and differentiation. Employing such primers, PCR-based single-strand conformation polymorphism (SSCP) with selective sequencing of mt gene regions (with differing levels of

sequence variation) (cf. Gasser et al. 2006) would be well suited for this purpose; this approach has been applied previously to the bovine lungworm (e.g., Hu et al. 2004).

Because sequence variation in nuclear ribosomal DNA (e.g., second internal transcribed spacer, ITS-2) is usually low within most species of strongylid nematodes (cf. Gasser 2006; Gasser et al. 2006), mt DNA will likely be applicable for estimating population variation in *P. normani* and related species. Thus, having available the mt genome of *P. normani* provides a basis for using a PCR-based analytical approach, whereby mt genetic markers, such as *cox1*, *nad1* and *nad4* (displaying varying levels of within-species divergence) are employed to estimate haplotypic variability in *P. normani* populations in Fur Seals (definitive host) and match sequences to those detected in potential intermediate hosts. Given that species complexes are commonly encountered in strongylid nematodes (Blouin 2002; Gasser 2006), it would be interesting to prospect for cryptic species, evaluate whether various haplotypes of *P. normani* exist and assess whether they might have particular host preferences and cause distinct clinical entities of lung disease in seals, and whether particular subpopulations of *P. nomani* might differ in their transmission patterns. Given the adverse impact of lungworm and associated secondary bacterial infections can have on seal health (cf. Lehnert et al. 2010), there is an imperative in identifying and tracking *Parafilearoides* spp. throughout their life cycles.

Once mt genomic datasets are available for a wide range of lungworms, it should be possible to comprehensively reassess the systematic relationships of lungworms (order Strongylida) using inferred amino acid data sets, logically extending some previous studies of lungworms (e.g., Carreno and Nadler 2003; Chilton et al. 2006; Lehnert et al. 2010). It is evident that there has been considerable controversy as to the position of *Parafilearoides* species in relation to members of the Filaroididae and the Pseudoliidae. Originally, *Parafilearoides* spp. were considered to be similar to *Filaroides* spp. but different in having indistinct (or degenerate) papillary rays and in having smaller spicules than those of

Filaroides spp. (Dougherty 1946). Some descriptions of *Parafilaroides* spp, such as that of *P. gymunrus*, have indicated significant challenges in identifying and classifying these worms, because of their delicate and indistinct morphological features (Gosselin and Measures 1997). Although *P. gymunrus* was first described as a species of *Pseudalius*, the morphological affinity of *Parafilaroides* spp. to members of the Pseudoliidae is not apparent, as most members of this family have distinct features at their caudal ends (Arnold and Gaskin 1975), although some representatives have greatly reduced bursa (Dougherty 1944). Nonetheless, molecular findings (using nuclear ribosomal DNA data sets) support the proposal that *Parafilaroides* spp. (e.g., *P. decorus*) are more closely related to the Pseudoliidae than the Filaroididae, and that the Filaroididae are polyphyletic, suggesting that the loss of the bursa occurred independently during evolution (Carreno and Nadler 2003). Clearly, the high phylogenetic signal and consistent evidence of high nodal support values achieved using complete mt amino acid data sets in recent phylogenetic analyses of nematodes (e.g., Park et al. 2011; Jex et al. 2010b; Jabbar et al. 2013b) indicate that this approach should allow this hypothesis to be independently assessed, provided a sufficient number of appropriate lungworm taxa are included in a reconstruction.

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Fig. 1 Schematic representation of the circular mt genome of *Parafilaroides normani*. Individual transfer RNA genes are identified by one-letter amino acid codes (arrow). All genes are transcribed in the clockwise direction.

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370 **Table 1**

371 Nucleotide composition (%) of the entire mt genome of *Parafilaroides normani* and of
 372 individual protein and RNA genes within this genome.

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<i>Nucleotide</i>	<i>Length (bp)</i>	<i>A</i>	<i>C</i>	<i>T</i>	<i>G</i>	<i>A+T</i>
Entire sequence	13411	21.0	6.5	51.2	21.3	72.2
Protein genes	10344	18.6	6.5	52.8	22.1	71.4
RNA genes	1661	27.8	7.0	46.7	18.5	74.5

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Table 2

POSITIONS AND nucleotide sequence lengths of individual genes in the mt genome of *Parafilaroides normani* as well as codons (start and stop), anticodons and the lengths of predicted proteins.

Gene	nt positions	Sequence Lengths		Codons		Anticodon
		No. of nt	No. of aa	Start	Stop	
<i>cox1</i>	1 – 1572		522	ATA	TAG	
<i>trnC</i>	1572 – 1626	55				GCA
<i>trnM</i>	1625 – 1685	60				CAT
<i>trnD</i>	1701 – 1755	55				GTC
<i>trnG</i>	1756 – 1811	56				TCC
<i>cox2</i>	1812 – 2507		230	ATT	TAG	
<i>trnH</i>	2506 – 2559	53				GTG
<i>rrnL</i>	2560 – 3521	961				
<i>nad3</i>	3516 – 3851		99	ATT	TAG	
<i>nad5</i>	3853 – 5422		472	ATT	TAA	
<i>trnA</i>	5422 – 5476	54				TGC
<i>trnP</i>	5659 – 5716	57				TGG
<i>trnV</i>	5713 – 5765	53				TAC
<i>nad6</i>	5776 – 6207		142	TTG	TAG	
<i>nad4L</i>	6209 – 6440		82	ATT	TAA	
<i>trnW</i>	6437 – 6493	57				TCA
<i>trnE</i>	6494 – 6547	54				TTC
<i>rrnS</i>	6550 – 7246	696				
<i>trnS</i> (UCN)	7245 – 7299	54				GCT
<i>trnN</i>	7297 – 7351	55				GTT
<i>trnY</i>	7355 – 7410	56				GTA
<i>nad1</i>	7433 – 8287		314	ATT	TAG	
<i>atp6</i>	8292 – 8885		196	ATG	TAG	
<i>trnK</i>	8882 – 8940	59				TTT
<i>trnL</i> (UUR)	8944 – 8998	55				TAA
<i>trnS</i> (AGN)	8999 – 9050	51				TGA
<i>nad2</i>	9039 – 9884		289	TTG	TAG	
<i>trnI</i>	9898 – 9958	61				GAT
<i>trnR</i>	9963 – 10016	53				ACG
<i>trnQ</i>	10016 – 10069	54				TTG
<i>trnF</i>	10079 – 10135	57				GAA
<i>ctyb</i>	10146 – 11246		366	ATG	TAG	
<i>trnL</i> (CUN)	11242 – 11295	54				TAG
<i>cox3</i>	11285 – 12070		260	ATG	TAA	
<i>trnT</i>	12062 – 12113	52				TGT
<i>nad4</i>	12091 – 13311		405	ATT	TAA	

Table 3

Codon usages (%) in mt protein genes of *Parafilaroides normani*. International Union of Pure and Applied Chemistry (IUPAC) codes (N = A, G, C or T; Y = C or T; R = A or G) were used

<i>Amino acid</i>	<i>Codon</i>	<i>Number of codons</i>	<i>Codons usage (%)</i>
Non-polar			
Alanine	GCN	82	2.4
Isoleucine	ATY	242	7.0
Leucine	CTN	324	9.4
Leucine	TTR	527	15.3
Methionine	ATR	128	3.7
Phenylalanine	TTY	561	16.3
Proline	CCN	75	2.2
Tryptophan	TGR	53	1.5
Valine	GTN	332	9.6
Total		1676	48.7
Polar			
Asparagine	AAY	88	2.6
Cysteine	TGY	64	1.9
Glutamine	CAR	37	1.1
Glycine	GGN	204	5.9
Serine	AGN	243	7.1
Serine	TCN	157	4.6
Threonine	ACN	80	2.3
Tyrosine	TAY	178	5.2
Total		415	12.1
Acidic			
Aspartate	GAY	73	2.1
Glutamate	GAR	77	2.2
Total		150	4.4
Basic			
Arginine	CGN	125	3.6
Histidine	CAY	56	1.6
Lysine	AAR	85	2.5
Total		266	7.7

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