Research article

The mitochondrial genomes of *Ancylostoma caninum* and *Bunostomum phlebotomum* – two hookworms of animal health and zoonotic importance

Aaron R Jex¹, Andrea Waeschenbach*², Min Hu¹, Jan A van Wyk³, Ian Beveridge¹, D Timothy J Littlewood² and Robin B Gasser¹

Address: ¹Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia, ²Department of Zoology, The Natural History Museum, Cromwell Road, London, UK and ³Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, 0110 Onderstepoort, South Africa

Email: Aaron R Jex - ajex@unimelb.edu.au; Andrea Waeschenbach* - a.waeschenbach@nhm.ac.uk; Min Hu - mhu@unimelb.edu.au; Jan A van Wyk - janvwyk@op.up.ac.za; Ian Beveridge - ibeve@unimelb.edu.au; D Timothy J Littlewood - t.littlewood@nhm.ac.uk; Robin B Gasser - robinbg@unimelb.edu.au

* Corresponding author

Abstract

**Background:** Hookworms are blood-feeding nematodes that parasitize the small intestines of many mammals, including humans and cattle. These nematodes are of major socioeconomic importance and cause disease, mainly as a consequence of anaemia (particularly in children or young animals), resulting in impaired development and sometimes deaths. Studying genetic variability within and among hookworm populations is central to addressing epidemiological and ecological questions, thus assisting in the control of hookworm disease. Mitochondrial (mt) genes are known to provide useful population markers for hookworms, but mt genome sequence data are scant.

**Results:** The present study characterizes the complete mt genomes of two species of hookworm, *Ancylostoma caninum* (from dogs) and *Bunostomum phlebotomum* (from cattle), each sequenced (by 454 technology or primer-walking), following long-PCR amplification from genomic DNA (~20–40 ng) isolated from individual adult worms. These mt genomes were 13717 bp and 13790 bp in size, respectively, and each contained 12 protein coding, 22 transfer RNA and 2 ribosomal RNA genes, typical for other secernentean nematodes. In addition, phylogenetic analysis (by Bayesian inference and maximum likelihood) of concatenated mt protein sequence data sets for 12 nematodes (including *Ancylostoma caninum* and *Bunostomum phlebotomum*), representing the Ascaridida, Spirurida and Strongylida, was conducted. The analysis yielded maximum statistical support for the formation of monophyletic clades for each recognized nematode order assessed, except for the Rhabditida.

**Conclusion:** The mt genomes characterized herein represent a rich source of population genetic markers for epidemiological and ecological studies. The strong statistical support for the construction of phylogenetic clades and consistency between the two different tree-building methods employed indicate the value of using whole mt genome data sets for systematic studies of nematodes. The grouping of the Spirurida and Ascaridida to the exclusion of the Strongylida was not supported in the present analysis, a finding which conflicts with the current evolutionary hypothesis for the Nematoda based on nuclear ribosomal gene data.

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Background

Hookworms (Nematoda: Strongylida: Ancylostomatidae) are blood-feeding nematodes that inhabit the small intestines of their mammalian host. Species of Ancylostoma, Necator, Bunostomum and Globocephalus, for instance, are of major human or animal health significance in various countries [1-6]. The infective, third-stage larvae (L3) can be ingested or penetrate the skin of the host and migrate via the circulatory system and the lungs to finally reside, as dioecious adults, usually in the duodenum. The adults attach via their buccal capsule to the intestinal mucosa, rupture capillaries and feed on blood. The pathogenesis of hookworm disease in humans and other animals is mainly a consequence of the blood loss, which occurs during parasite attachment and feeding in the intestine. Cutaneous infection can occur and is often associated with inflammatory/immune responses and painful, eruptive lesions during the migration of larvae through the skin [7,8].

Current estimates indicate that more than 740 million people are infected with the hookworms Ancylostoma duodenale and Necator americanus [9], and ~80 million are severely clinically affected by hookworm disease [10]. In a large number of developing countries, hookworms are a leading cause of iron deficiency anaemia, which, in heavy infections, can cause physical and mental retardation and deaths in children as well as adverse maternal-fetal outcomes [10,11]. Although there is considerably less information on the prevalence and geographical distribution of hookworms of animals [7,12-15], these parasites are also clinically important in dogs (Ancylostoma braziliense, Ancylostoma caninum, Ancylostoma ceylanicum and Uncinaria stenocephala), cats (Ancylostoma tubaeforme), ruminants (Bunostomum phlebotomum, Bunostomum trigonocephalum and Gaigeria pachyceles), pigs (e.g., Globocephalus urosubulatus) and other hosts [16]. Hookworms were originally thought to be host-specific [17,18]; however, the canine hookworm, Ancylostoma caninum, for example, can infect humans and cause dermatitis and eosinophilic enteritis [19], and some hookworm species, such as the bovine hookworm, Bunostomum phlebotomum, have been linked to cutaneous lesions in humans [20]. Significant genetic variation has been described among individuals of Ancylostoma caninum from dogs in Australia [21]. Such variation might reflect differences in host specificity, infectivity and/or pathogenicity among individual nematodes within a population or, in some cases, might be indicative of speciation events, as has been hypothesized previously for human hookworms [21,22]. Presently, there are no published studies of genetic variation within and among populations of Bunostomum phlebotomum and no molecular data are publicly available for this species.

The ability to accurately identify hookworms to species and to assess genetic variability in hookworm populations is central to studying their epidemiology as well as to diagnosis and control. Sequences of the first and second intragenomic transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) [23-25] and of cAMP-dependent protein kinase [26] have been utilized to identify and differentiate hookworm species. However, the ITS-1 and ITS-2 regions do not usually display sufficient within-species sequence variability to enable the study of the genetic structuring within and among hookworm populations [24]. In contrast, mitochondrial (mt) genomes have been shown to contain useful genetic markers for studying the population structures of hookworm species [27-31], because of their rapid mutation rates and apparent maternal inheritance [32-34]. Although the protein-coding mt gene cytochrome c oxidase subunit 1 (cox1) is applicable to population studies of a range of invertebrates, including parasitic platyhelminths [35,36] and some nematodes [37,38], there are still limited sequence data for cox1 and other mt genes of hookworms, and limited published information is available on sequence heterogeneity therein. Building on advances in long polymerase chain reaction (PCR)-based mt genome sequencing [39-41], the present study determined the sequences and structures of the two mt genomes from an individual of Ancylostoma caninum (from a dog) from Australia and a specimen of Bunostomum phlebotomum (from a calf) from South Africa. The sequences derived for the mt genomes of these two hookworms were compared in detail with mt genomic data available for the predominant hookworms of humans, Ancylostoma duodenale and Necator americanus [42], as well as those available for other selected species belonging to the orders Strongylida [41], Ascaridida [43-45] and Spirurida [46-48].

Results and Discussion

Mitochondrial genome features, characteristics and gene organization

The circular mt genomes of Ancylostoma caninum and Bunostomum phlebotomum, sequenced from single adult worms, were 13717 and 13790 bp in size, respectively (Figure 1). Each genome contained 36 genes, including 12 protein coding genes (adenosine triphosphatase subunit 6 [atp6], the cytochrome c oxidase subunits [cox1-3], cytochrome b [cytb], and the nicotinamide dehydrogenase subunits [nad1-6 and nad4l]), 22 transfer RNA (tRNA) genes and 2 ribosomal RNA genes (small [rrn6] and large [rrn16] subunits), and was consistent with gene arrangement 2 (GA2) [49]. This arrangement is characteristic for the mt genomes of all members of the Strongylida and Ascaridida, as well as the free-living nematode Caenorhabditis elegans (Rhabditida), but not for Strongyloides stercoralis (Rhabditida) [41-49]. In accordance with other species of Strongylida for which complete mt genome
The A+T content for *rrnS*, *rrnL* (= ribosomal RNA genes) and the AT-rich region were 78.1%, 80.9% and 90.1%, respectively, for *Ancylostoma caninum*, and 75.2%, 82.4% and 88.0%, respectively, for *Bunostomum phlebotomum*. For the mt genome of *Ancylostoma caninum*, codon usage in individual protein coding genes (n = 12) ranged from 0% for CGC (arginine) and CCC (proline) to 15.7% for TTT (phenylalanine). For the mt genome of *Bunostomum phlebotomum*, codon usage ranged from 0% for CGC (arginine), CAC (histidine), CTC (leucine), CCC (proline), TCC (serine) and GTC (valine) to 15.0% for TTT (phenylalanine). For both species, individual tRNA structures were consistent with those predicted previously for hookworms and other secernentean nematodes [37,42,45,50,51]. All tRNA genes, except *trnS*(AGN) and *trnS*(UCN), had a predicted secondary structure containing a TV-replacement loop instead of the TyC arm and loop (not shown). The predicted secondary structure of each of the two serine tRNAs contained the TyC arm and loop but lacked the DHU loop. The genes *rrnS* and *rrnL* were 694 bp and 935 bp in length, respectively; the predicted secondary structures for the ribosomal RNA gene subunits for *Ancylostoma caninum* and *Bunostomum phlebotomum* (not shown) were similar to those of *Necator americanus* and *Ancylostoma duodenale* [42], which is also supported by the high nucleotide sequence similarity in the mt genes among these four hookworms (see Tables 2 and 3).

The AT-rich regions for *Ancylostoma caninum* and *Bunostomum phlebotomum* were 272 bp and 234 bp, respectively, and both exhibited complex secondary structures (not shown), as predicted previously for the AT-rich regions of nematodes [41,42,45,47,49]. Four AT-repeat regions of variable length were identified in the AT-rich region of the mt genome of *Ancylostoma caninum*: two were 6 nucleotides (nt) (3 AT-repeats), one was 14 nt (7 AT-repeats) and the longest was 16 nt (8 AT-repeats). Similar dinucleotide repeats have been described in the AT-rich region of the mt genomes of other nematode species (e.g., [41,42,44]). Other repetitive elements have been identified within this region in the free-living nematode *Caenorhabditis elegans*, the largest and most conspicuous of which are the repetitive sequence motifs CR1-CR6 [45]. However, no such elements were identified in the AT-rich region of the mt genome of either *Ancylostoma caninum*, *Bunostomum phlebotomum* or any other species of animal-parasitic nematode sequenced to date [41,44,47,49].

**Comparative analyses with other nematodes**

The identities (%) in inferred amino acid sequences of each protein-coding mt gene were calculated based upon pairwise comparisons between *Ancylostoma caninum* and *Bunostomum phlebotomum* (Tables 2 and 3). Based on these comparisons, the sequence identities (in decreasing
order) were COX1 (97.3%), COX3 (96.0%), NAD4L (93.5%), COX2 (95.6%), CYTB (87.0%), ATP6 (85.9%), NAD1 (84.4%), NAD3 (81.9%), NAD4 (79.2%), NAD5 (78.5%), NAD2 (72.5%) and NAD6 (67.3%). In addition, the amino acid sequences inferred from each coding mt gene of Ancylostoma caninum and Bunostomum phlebotomum were compared, again in a pairwise manner, with those inferred from published mt genomes of Anisakis simplex [43], Ascaris suum [45] and Toxocara canis [44] (Ascaridida), Ancylostoma duodenale, Necator americanus [42] and Haemonchus contortus [41] (Strongylida), and Brugia malayi [46], Dirofilaria immitis [47] and Onchocerca volvulus [48] (Spirurida). The most conserved protein sequences among all species, assessed relative to Ancylostoma caninum and Bunostomum phlebotomum, were inferred to be COX1, COX3 and NAD4L, and the least conserved were NAD2 and NAD6 (see Tables 2 and 3).

Phylogenetic analyses of selected species of Ascaridida, Spirurida and Strongylida using concatenated amino acid sequence data inferred from mt genes

Because of the high degree of intraspecific variation in nucleotide sequence in the mt genes of nematodes [37,38,52] and the limited availability or lack of multiple

Table 1: Nucleotide (nt) composition (%) and A+T contents (%) of the 12 mitochondrial protein coding genes.

| Gene or region | Ancylostoma caninum | Bunostomum phlebotomum |
|---------------|---------------------|-------------------------|
| Length (nt)   |                     |                         |
| ATP6          | 600                 | 600                     |
| COX1          | 1578                | 1578                    |
| COX2          | 696                 | 696                     |
| COX3          | 766                 | 766                     |
| CYTB          | 1113                | 1113                    |
| NAD1          | 870                 | 870                     |
| NAD2          | 846                 | 846                     |
| NAD3          | 336                 | 336                     |
| NAD4          | 1230                | 1230                    |
| NAD4L         | 234                 | 234                     |
| NAD5          | 1582                | 1582                    |
| NAD6          | 432                 | 432                     |
| rrnL          | 963                 | 963                     |
| rrnS          | 694                 | 694                     |
| AT-rich       | 272                 | 272                     |
| Genome        | 13735               | 13790                   |

In addition: nucleotide sequences for each of the ribosomal RNA genes of Ancylostoma caninum (Ac) and Bunostomum phlebotomum (Bp) with those published for species of Strongylida [Ancylostoma duodenale (Ad), Necator americanus (Na) and Haemonchus contortus (Hc)], species of Ascaridida [Anisakis simplex (Ans), Ascaris suum (Ass) and Toxocara canis (Tc)] and species of Spirurida [Brugia malayi (Bm), Dirofilaria immitis (Di) and Onchocerca volvulus (Ov)].

Table 2: Pairwise comparison (%) of the amino acid sequences inferred for each of the mitochondrial protein coding genes.

| Protein or gene | Bp  | Ad  | Na  | Hc  | Ans | Ass | Tc  | Bm  | Di  | Ov  |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ATP6          | 85.9| 98.4| 89.4| 82.4| 76.3| 77.3| 76.8| 19.8| 22.2| 21.2|
| COX1          | 97.3| 99.2| 97.1| 94.4| 89.5| 90.4| 91.2| 50.7| 50.9| 51.4|
| COX2          | 89.6| 96.1| 92.6| 88.3| 83.1| 83.6| 84.4| 41.0| 42.3| 41.0|
| COX3          | 96.0| 99.2| 95.2| 91.0| 81.5| 81.9| 84.7| 33.9| 33.5| 32.0|
| CYTB          | 87.0| 98.1| 85.9| 81.6| 70.8| 74.5| 73.5| 51.0| 48.9| 50.8|
| NAD1          | 84.4| 95.1| 87.9| 75.5| 70.0| 71.7| 70.8| 46.8| 50.6| 49.1|
| NAD2          | 72.5| 90.0| 77.9| 51.6| 48.9| 46.5| 51.2| 33.3| 28.7| 32.0|
| NAD3          | 81.9| 96.3| 81.0| 69.3| 67.5| 68.4| 66.6| 35.7| 35.7| 35.7|
| NAD4          | 79.2| 95.1| 85.8| 73.1| 64.3| 63.5| 63.0| 45.2| 44.9| 45.8|
| NAD4L         | 93.5| 100.0| 93.5| 69.6| 75.3| 72.7| 68.8| 35.0| 29.1| 37.5|
| NAD5          | 78.5| 94.3| 83.6| 71.1| 66.0| 63.4| 65.5| 38.9| 37.4| 39.3|
| NAD6          | 67.3| 88.8| 70.8| 51.3| 59.0| 56.2| 51.3| 26.4| 26.4| 27.1|
| rrnL          | 83.4| 91.6| 82.5| 75.4| 70.5| 70.0| 66.4| 62.6| 61.3| 60.4|
| rrnS          | 86.8| 94.9| 86.5| 78.7| 73.0| 73.3| 70.9| 60.6| 60.6| 59.4|

In addition: nucleotide sequences for each of the 12 mitochondrial protein coding genes of Ancylostoma caninum (Ac) and Bunostomum phlebotomum (Bp) with those published for species of Strongylida [Ancylostoma duodenale (Ad), Necator americanus (Na) and Haemonchus contortus (Hc)], species of Ascaridida [Anisakis simplex (Ans), Ascaris suum (Ass) and Toxocara canis (Tc)] and species of Spirurida [Brugia malayi (Bm), Dirofilaria immitis (Di) and Onchocerca volvulus (Ov)].
Table 3: Pairwise comparison (%) of the amino acid sequences inferred for each of the mitochondrial protein coding genes and nucleotide sequences for each of the ribosomal RNA genes of *Ancylostoma caninum* (Ac) and *Bunostomum phlebotomum* (Bp).

| Protein or gene | Ac  | Ad  | Na  | Hc  | Ans | Ass | Tc  | Bm  | Di  | Ov  |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ATP6           | 85.9| 85.4| 86.9| 78.8| 73.3| 74.3| 74.8| 19.8| 21.7| 21.2|
| COX1           | 97.3| 96.9| 97.3| 94.2| 89.7| 90.8| 91.6| 50.7| 50.9| 51.4|
| COX2           | 89.6| 88.3| 92.2| 87.4| 79.7| 82.7| 81.8| 41.4| 41.8| 40.5|
| COX3           | 96.0| 95.6| 94.5| 91.8| 80.7| 82.3| 83.5| 33.2| 33.2| 32.0|
| CYTB           | 87.0| 87.0| 83.7| 79.1| 69.4| 73.2| 72.4| 53.2| 51.6| 53.5|
| NAD1           | 84.4| 84.4| 84.8| 74.4| 69.3| 69.6| 69.5| 48.4| 52.3| 50.1|
| NAD2           | 72.5| 71.1| 69.7| 50.1| 53.5| 48.1| 52.2| 35.4| 30.8| 35.2|
| NAD3           | 81.9| 81.9| 75.6| 67.5| 64.8| 67.5| 63.9| 38.3| 37.5| 39.2|
| NAD4           | 79.2| 79.4| 80.6| 69.6| 67.4| 65.0| 65.0| 45.2| 44.4| 44.1|
| NAD4L          | 93.5| 93.5| 92.2| 65.8| 71.4| 68.8| 64.9| 30.3| 37.5| 33.7|
| NAD5           | 78.5| 77.9| 79.1| 67.3| 63.2| 64.7| 64.5| 42.1| 39.1| 39.9|
| NAD6           | 67.3| 70.1| 70.8| 48.6| 58.3| 57.6| 52.7| 27.8| 28.4| 29.8|
| rrmL           | 83.4| 82.6| 80.2| 73.9| 69.2| 70.1| 67.5| 63.9| 62.8| 61.6|
| rrmS           | 86.8| 85.6| 86.0| 75.6| 74.5| 74.1| 70.6| 61.1| 61.3| 60.5|

With those published for species of Strongylida [*Ancylostoma duodenale* (Ad), *Necator americanus* (Na) and *Haemonchus contortus* (Hc)], species of Ascaridida [*Anisakis simplex* (Ans), *Ascaris suum* (Ass) and *Toxocara canis* (Tc)] and species of Spirurida [*Brugia malayi* (Bm), *Dirofilaria immitis* (Di) and *Onchocerca volvulus* (Ov)].

mt genome sequences for each species, previous work has suggested that phylogenetic analyses for nematodes be conducted using concatenated amino acid sequence data sets, utilizing sequences inferred from individual mt protein coding genes [47]. In order to further assess systematic relationships within and among members of the Ascaridida, Spirurida and Strongylida, a phylogenetic analysis was carried out using Bayesian inference (BI) and maximum likelihood (ML) (Figure 2). Almost all clades in the consensus tree were supported by maximum BI posterior probability (pp) values (pp = 1.00; expressed as a percentage in Figure 2) and/or ML bootstrap support (100). The phylogenetic analysis conducted herein clearly supports the distinct classification of the orders Ascaridida, Spirurida and Strongylida, each as monophyletic clades with maximum statistical support. The order Rhabditida appears to be paraphyletic, with *Caenorhabditis elegans* grouping closely with the Strongylida, and *Steinernema carpocapsae* and *Strongyloides stercoralis* placed externally to a clade comprising the Ascaridida, Strongylida and *C. elegans*. This relationship is consistent with the proposed molecular phylogeny for the Nematoda based on small subunit (18S) nuclear ribosomal DNA data [53]. In addition, the hookworms were represented as a monophyletic clade within the Strongylida.

For hookworms, the phylogenetic analysis using BI indicated a closer relationship between *Ancylostoma* spp. and *Necator americanus* than between either of them and *Bunostomum phlebotomum*. This finding conflicts with the current classification of the Strongylida [16], wherein both *Necator* and *Bunostomum* are placed within the subfamily Bunostominae, whereas *Ancylostoma* is placed within the subfamily Ancylostominae (poorly supported by the ML analysis; bootstrap support = 47). A larger analysis, including mt data for more hookworm species, is needed to test further this hypothesis.

The present phylogenetic analysis did not support the grouping of the Ascaridida and Spirurida to the exclusion of the Strongylida.

Figure 2
Phylogenetic analysis (using Bayesian inference) of concatenated mt amino acid sequence data inferred from all protein coding mitochondrial genes (n = 12) for 16 secernentean nematodes, including *Ancylostoma caninum* and *Bunostomum phlebotomum* (GenBank accession numbers FJ483518 and FJ483517, respectively). The concatenated mitochondrial amino acid sequence of three mermithids were employed as outgroups. Bayesian posterior probability values (as a percentage) and maximum likelihood bootstrap support (n = 100) are indicated above and below the lines, respectively. The scale indicates an estimate of substitutions per site, using the optimized model setting.
of the Strongylida, which contrasts markedly the results of a previous study based on nuclear ribosomal gene data (e.g., clade III versus clade V; ref. [53]). The "common heritage" hypothesized herein for the Ascaridida and Strongylida to the exclusion of the Spirurida has been supported by previous studies using mt gene order data [49] and using concatenated amino acid sequence data inferred from protein-coding mt genes [38]. These findings stimulate further study of the evolutionary relationships among taxa within this phylum using mt datasets. The high-throughput sequencing potential of 454 technology [54] and the recent validation of this technique for the sequencing of mt genomes [41] should provide a platform for an in-depth analysis of the phylogeny of the Nematoda.

Conclusion

Utility of mt gene markers for population genetic, ecological and epidemiological studies of hookworms

Although some nuclear genetic regions (e.g., ITS-1 and ITS-2 of nuclear rDNA [22-25] or the cAMP-dependent protein kinase gene [26]) have been shown to be suitable for the specific identification and differentiation of hookworms, the nuclear loci examined to date do not usually display sufficient levels of intraspecific sequence variability for the investigation of the genetic structures of hookworm populations (or the identification of population variants or "strains"). The ability to estimate genetic variability within and among hookworm populations is central to studying their epidemiology and population genetics, and can have important practical implications in relation to control.

Sequence-based analyses (including mutation scanning) of protein-coding mt genes, such as cox1 and nad1, have been particularly useful or population genetic studies [21,27,29-31,55-59]. For example, Hu et al. [21] employed a single-strand conformation polymorphism (SSCP)-coupled sequencing approach to explore haplotypic variability within a limited number of Ancylostoma caninum specimens from Australia and each of the human hookworms (Ancylostoma duodenale and Necator americanus). Significant population sub-structuring was recorded within each of these three species, and two genetically distinct subpopulations were detected within Ancylostoma caninum from dogs from Townsville, Australia. Previous morphological and clinical studies had shown that Ancylostoma caninum in Townsville (Australia) is not specific to dogs and can also infect humans (but not complete its life-cycle), causing eosinophilic enteritis [19]. It has been speculated [21] that particular, genetically distinct subpopulations within Ancylostoma caninum can selectively infect the non-canine host. The pattern of haplotypic variability within Ancylostoma caninum might be due to secondary contact between populations or subpopulations, which could have arisen due to host movement from other geographical areas where this hookworm has been recorded and where ecological conditions are distinct; for example, Ancylostoma caninum is endemic in tropical north-east Queensland, Australia [60], but also occurs in the north-west area of Western Australia [61]. It is also possible that feral dogs or dingoes (in different geographical or climatic regions) might harbour one or more genetic variants which might "spill-over" into domestic dogs and/or humans [60]. Future study of the genetic variation among Ancylostoma caninum specimens from domestic and feral dogs, cats and humans as well as between populations from other geographical and climatic regions in Australia and South-East Asia would allow such questions to be addressed. A comparison of the genetic make-up of Ancylostoma caninum from humans affected by eosinophilic enteritis with those from domestic dogs in the Townsville area would be particularly interesting.

In contrast to Ancylostoma caninum, no studies have yet explored the genetics or molecular epidemiology of Bunostomum phlebotomum. Mitochondrial markers might be used to examine sub-structuring in Bunostomum phlebotomum populations in endemic regions of South Africa. In addition, although there has been anecdotal evidence suggesting that Bunostomum phlebotomum may cause cutaneous larval migrans in humans ([20] and unpublished observations [JVW]), the zoonotic potential of this species of hookworm has not yet been tested molecularly. In view of the lack of distinguishing morphological characters allowing the identification of individual larvae, the provision of molecular markers for Bunostomum phlebotomum might allow the extent of the zoonotic potential of this species to be assessed for the first time.

The two mt genomes characterized herein provide a solid foundation for studies of the epidemiology, ecology and population genetics of both Ancylostoma caninum and Bunostomum phlebotomum, which could have important implications for the control of infections by these parasites. Given the lack of morphological characters for specific identification and differentiation of hookworm larvae, there is a clear need for species and population genetic markers for in-depth exploration of the epidemiology of hookworms [59]. Combined with the use of specific markers in the internal transcribed spacers (ITS-1 and ITS-2) of nuclear rDNA [23-25], investigating the mt haplotypic variability in populations of Ancylostoma caninum and Bunostomum phlebotomum (irrespective of developmental stage) could provide important insights into host affiliations, gene flow and transmission patterns (cf. [62,63]) and thus assist in the control of these hookworms. Furthermore, the direct sequencing of the mt genome of Ancylostoma caninum by 454 technology is the
second example of the use of this approach for the sequencing of mt genomes of nematodes [41] and reinforces the exciting potential of emerging technologies for the high-throughput sequencing of relatively small organellar genomes.

Methods
Parasites and DNA extraction
An adult male of *Ancylostoma caninum* (designated Ac1) was collected (by IB) at necropsy from the duodenum of a dog from Townsville, Australia [23]. An adult male of *Bunostomum phlebotomum* (Bp1) was collected at autopsy from the same site from a calf monospecifically infected with an isolate of *Bunostomum phlebotomum*, originally derived from a Jersey cow in Pretoria North suburb, South Africa (by JvW). Nematodes were washed in physiological saline, identified morphologically to species [16], fixed in 50% (v/v) ethanol and stored at -20°C until use. Total genomic DNA was isolated from individual worms using sodium dodecyl-sulphate/proteinase K treatment [64], followed by spin-column purification (Wizard Clean-Up, Promega). The specific identity of each nematode was verified using the sequence of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA, which provides species-specific genetic markers for hookworms [25]. The ITS-2 sequence derived from sample Ac1 was identical to that reported previously for *Ancylostoma caninum* (accession number AJ001591) [25] and that obtained from Bp1 (accession number FJ616999) was 82.3% identical to the closely related species *Bunostomum trigonocephalum* (accession number A0617595) [25].

Long PCR-coupled mt genome sequencing
The complete mt genome of each *Ancylostoma caninum* and *Bunostomum phlebotomum* was amplified as two overlapping amplicons (~10 kb and ~5 kb, respectively) from ~20–40 ng of the genomic DNA from each specimen by long-PCR (BD Advantage 2, BD Biosciences) using each of the primer pairs 39F-42R and 5F-40R [39,40,42], as described by Hu et al. [39], with minor modifications. The cycling conditions (2720 thermal cycler, Applied Biosystems) were: 92°C, 2 min (initial denaturation); then 92°C, 10 s (denaturation); 50°C, 30 s (annealing); 68°C (for the ~10 kb region) or 60°C (for the ~5 kb region), 10 min (extension) for 10 cycles, followed by 92°C, 10 s; 50°C, 30 s; 68°C or 60°C, 10 min for 20 cycles, with an elongation period of 10 s for each cycle, and a final extension at 68°C or 60°C for 7 min. Following the PCR, individual amplicons were resolved in ethidium bromide-stained agarose (1%) gels and shown to represent single bands. Amplicon size was estimated based on comparison with a 1 kb DNA size ladder (Promega). Amplicons of ~10 kb or ~5 kb were purified over a mini-column (Wizard PCR Preps, Promega). Subsequently, the amount of DNA in each purified amplicon was estimated spectrophotometrically (ND-1000 UV-VIS spectrophotometer, v.3.2.1, NanoDrop Technologies). The purified amplicons were then subjected to sequencing.

The mt genome of *Ancylostoma caninum* (designated AcMG-454; GenBank accession no. FJ483518) was sequenced (454 technology by Al/RBG) using a Genome Sequencer 20 (Roche), according to an established protocol [54]. The complete mt genome of *Bunostomum phlebotomum* (designated BpMG-PW; GenBank accession no. FJ483517) was sequenced (AW/DTL) by primer walking, as described previously [44]. The AcMG-454 sequence was assembled automatically, whereas that of BpMG-PW was assembled manually using Sequencher v.4.8 (Gene Codes Corporation). Both mt genome sequences were annotated and subjected to analysis using standard approaches [41,44], and their structures were compared with each other and with those of the two human hookworms, *Ancylostoma duodenale* (GenBank accession number AY117719; ref. [42] and *Necator americanus* (AY117720; ref. [42], *Haemonchus contortus* (EU346694; ref. [41]) (Strongyldida); Anisakis simplex (AY994157; ref. [43], *Acaris suum* (X53453; ref. [45]), and *Toxocara canis* (EU730761; ref. [44]) (Ascaridida); *Brugia malayi* (AF538716; ref. [46]), *Dirofilaria immitis* (AF537512; ref. [47]) and *Onchocerca volvulus* (AF015193; ref. [48]) (Spirurida).

Phylogenetic analysis
The analysis of amino acid sequence data was conducted via Bayesian inference (BI) using the software package MrBayes v.3.1.2 http:// mrbayes.csit.fsu.edu/index.php and maximum likelihood (ML) using GARLI ([65]; http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html), each running on a four-core Opteron-based Unix cluster. For individual species, the amino acid sequences inferred from all protein coding mt genes were concatenated. A selection of published mermithid mt genomes were used as outgroups (Strektovimermis spiculatus, accession NC_008047; Agamermis sp., NC_008231; Thaumermis cosgrovei, NC_008046) and included a range of ingroup taxa (Xiphinema americanum, NC_005928; Brugia malayi, NC_004298; Onchocerca volvulus, NC_001861; Dirofilaria immitis, NC_005305; Strongyloides stercoralis, NC_005143; Caenorhabditis elegans, NC_001328; Steinernema carpocapsae, NC_005941; Necator americanus, NC_003416; Ancylostoma duodenale, NC_003415; Cooperia oncophora, NC_004806; Haemonchus contortus, NC_010383; Anisakis simplex, NC_007934; Acaris suum, NC_003127; Toxocara canis, NC_010690). Amino acid sequences were aligned using MUSCLE [66]. Ambiguous sites were excluded using G-Blocks ([67,68]; see Additional File 1 for alignment).

For BI of amino acid data, tree construction and posterior probabilities (pp) were calculated via 2000000 genera-
tions (ngen = 2000000) using the Metropolis-coupled Monte Carlo Markov Chain (MCMC-MCMC) method and four simultaneous tree-building chains (nchains = 4), with every 10th tree being saved (samplefreq = 10). A suitable burnin (burnin = 1000) was chosen using ‘Trace’ in the program Tracer v.1.4 [http://beast.bio.ed.ac.uk/]. Evolutionary distance was estimated using the most appropriate amino acid model and calculated employing the MrBayes program (aamodelpr = mixed), allowing for a gamma-shaped variation in mutation rates with a proportion of invariant sites (rates = invgamma). Upon completion of the analysis, a 50% majority rule = consensus tree was constructed in TreeviewX v.0.5.0 [http://darwin.zool. gla.ac.uk/~rpage/treeviewx/]. For the ML analysis using GARLI, tree construction was estimated with the GTR+I+g model GTR+I+g using the mtRev amino acid substitution model. ARJ and RBG drafted the manuscript, analyses. IB and JvW provided morphologically-identified parasite material. ARJ and RBG conceived the study. ARJ and AW contributed equally to molecular work, assisted by MH. DTJL provided technical advice and conducted phylogenetic analyses. IB and JvW provided morphologically-identified parasite material. ARJ and RBG drafted the manuscript, with active inputs from all other authors. All authors approved the final manuscript.

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References
1. Miller TA: Vaccination against the canine hookworm diseases. Adv Parasitol 1971, 9:133-183.
2. Yu SH, Shen WY: Hookworm infection and disease in China. In Hookworm Disease: Current Status and New Directions Edited by: Schad G, Warren KS. London: Taylor and Francis; 1990:44-54.
3. Náquira C: Hookworm infection in Latin America and the Caribbean. In Hookworm Disease: Current Status and New Directions Edited by: Schad G, Warren KS. London: Taylor and Francis; 1990:55-68.
4. Kilama WL: Hookworm Infection and Disease in Africa and the Middle East. In Hookworm Disease: Current Status and New Directions Edited by: Schad G, Warren KS. London: Taylor and Francis; 1990:17-32.
5. Carroll SM, Walker JC: Hookworm infection in Southeast Asia, Oceania and Australia. In Hookworm Disease: Current Status and New Directions Edited by: Schad G, Warren KS. London: Taylor and Francis; 1990:33-43.
6. Yu SH, Jiang ZX, Xu LQ: Infantile hookworm disease in China. A review. Acta Trop 1995, 59(4):265-270.
7. Beveridge I: Australian hookworms (Ancylostomatoidae): a review of the species present, their distributions and biogeographical origins. Parasitology 2002, 124(1-2):63-88.
8. Heukelbach J, Feldmeier H: Epidemiological and clinical characteristics of hookworm-related cutaneous larva migrans. Lancet Infect Dis 2008, 8(5):302-309.
9. de Silva NR, Brooker S, Hotez PJ, Montessori A, Engels D, Savioi L: Soil-transmitted helminth infections: Updating the global picture. Trends Parasitol 2009, 19:547-551.
10. Hotez PJ, Bethony J, Bottazzi ME, Brooker S, Buss P: Hookworm: "The Great Infection of Mankind". PLoS Med 2005, 2(3):e67.
11. WHO: Deworming for Health and Development. Report of the Third Global Meeting of the Partners for Parasite Control 2005 [http://whqlibdoc.who.int/hq/2005/who_cds_cpe_pvc_2005.14.pdf].
12. Guest CM, Stephen JM, Price CJ: Prevalence of Campylobacter and four endoparasites in dog populations associated with Hearing Dogs. J Small Anim Pract 2007, 48(1):632-637.
13. Smith GC, Gangadharan B, Taylor Z, Laurenson MK, Bradshaw H, Hide G, Hughes JM, Dinkel A, Romig T, Craig PS: Prevalence of zoonotic important parasites in the red fox (Vulpes vulpes) in Great Britain. Vet Parasitol 2003, 118(1-2):133-142.
14. Yazywinski TA, Gibbs HC: Survey of helminth infections in Maine dairy cattle. Am J Vet Res 1975, 36(11):1677-1682.
15. Anderson TC, Foster GW, Forrester DJ: Hookworms of feral cats in Florida. Vet Parasitol 2003, 115(1):19-24.
16. Skrjabin KI, Shikhobalova NP, Schulz RS, Popova TI, Boev SN, Delyamure SL: Strongyloida, Keys to Parasitic Nematodes. In [English Translation of Opredelitel Paraziticheskikh Nematod. Izdatel'stvo Akademii Nauk SSSR, Moscow] Volume 3. Edited by: Skrjabin KI. American Publishing Co. Pvt. Ltd. 1992.
17. Biocca E: Rediscrizione di Ancylostoma tubaeforme (Zeder, 1800) parasita del gatto, considerato erroneamente sinonimo di Ancylostoma caninum (Ercoilan, 1859) parasita del cane. Riv Parasitol 1954, 15:267-278.
18. Burrows RB: Comparative morphology of Ancylostoma tubaeforme (Zeder, 1800) and Ancylostoma caninum (Ercoilan, 1859). J Parasitol 1962, 48:715-719.
19. Prociv P, Croese J: Human enteric infection with Ancylostoma caninum: hookworms reappeared in the light of a "new" zoonosis. Acta Trop 1996, 62(1):23-44.
20. Mayhew RL: Creeping eruption caused by the larvae of the cattle hookworm Bunostomum phlebotomum. Proc Soc Exp Biol Med 1947, 66:12-14.
21. Hu M, Chilton NB, Zhu X, Gasser RB: Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome c oxidase subunit I reveals significant substructuring in hookworm populations. Electrophoresis 2002, 23(1):27-34.
22. Rømsstad A, Gasser RB, Nansen P, Polderman AM, Chilton NB: *Necator americanus* (Nematoda: Ancylostomatidae) from Africa and Malaya have different ITS-2 rDNA sequences. *Int J Parasitol* 1998, 28(4):61-6-15.

23. Gasser RB, Stewart LE, Speare R: Genetic markers in ribosomal DNA for hookworm identification. *Acta Trop* 1996, 62(1):15-21.

24. Gasser RB, Monti JR, Bao-Zhen Q, Polderman AM, Nansen P, Chilton NB, A: A mutation scanning approach for the identification of hookworm species and analysis of population variation. *Mol Biochem Parasitol* 1998, 92(2):303-312.

25. Chilton NB, Gasser RB: Sequence differences in the internal transcribed spacers of DNA among four species of hookworm (*Ancylostoma*). *Int J Parasitol* 1999, 29(12):1971-1977.

26. Hawdon JM: Differentiation between the human hookworms *Ancylostoma duodenale* and *Necator americanus* using PCR-RFLP. *J Parasitol* 1996, 82(4):642-647.

27. Hu M, Chilton NB, Abs EL-Osta YG, Gasser RB: Long PCR amplification of mitochondrial genomes of nematode worms. *Mol Biochem Parasitol* 2004, 155:71-75.

28. Anderson TJ, Romero-Abal ME, Jaenike J: Comparative genomics of nematode mitochondria: idiosyncratic gene organization and evolutionary implications. *Int J Parasitol* 2003, 33(12):1391-1408.

29. Okimoto R, Wolstenholme DR: A set of tRNAs that lack either the T psi C arm or the dihydrouridine arm: towards a minimal tRNA adaptor. *Nucleic Acids Res* 1990, 18(24):7869-7874.

30. Brown WM: The mitochondrial genome of animals. In *Molecular Evolutionary Genetics* Edited by: MacIntyre RJ, New York: Plenum Press; 1985:95-130.

31. Miranda RR, Tennessen JA, Blouin MS, Rabelo EM: Mitochondrial DNA variation of the dog hookworm *Ancylostoma caninum*. *J Parasitol* 2007, 93(4):796-805.

32. Bowles J, McManus DP: Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol* 1992, 54(2):165-173.

33. Thompson RC, Meloni BP, Hopkins RM, Deplazes P, Reynoldson JA: Observations on the endo- and ectoparasites affecting dogs and cats in aboriginal communities in the north-west of Western Australia. *Aust Vet J* 1993, 70(7):268-272.

34. Kim KH, Eom KS, Park JK: The complete mitochondrial genome of *Anisakis simplex* (Ascaridida: Nematoda) and phylogenetic implications. *Int J Parasitol* 2006, 36:319-328.

35. Hedin E, Wang S, Sporo D, Caler E, Zhao Q, Crabtree J, Allen JE, Delcher AL, Guiliano DB, Miranda-Saavedra D, et al.: Draft genome of the filarial nematode parasite *Brugia malayi*. *Science* 2007, 317(5854):1756-1760.

36. Hu M, Gasser RB, Abs EL-Osta YG, Chilton NB: Structure and organization of the mitochondrial genome of the canine heartworm, *Dirofilaria immitis*. *Parasitology* 2003, 127(Pt 3):327-351.

37. Hu M, Chilton NB, Gasser RB: RT-PCR amplification using cDNA from *Ascaris suum* and *Necator americanus* for the identification of *Ancylostoma caninum*. *Submit* 2002, 21(10):2525-2526.
65. Zwickl DJ: Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. In PhD Dissertation Austin: University of Texas; 2006.

66. Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004, 32:1792-1797.

67. Castresana J: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 2000, 17:540-552.

68. Talavera G, Castresana J: Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 2007, 56:564-577.
Author/s:
Jex, AR; Waeschenbach, A; Hu, M; Van Wyk, JA; Beveridge, I; Littlewood, DTJ; Gasser, RB

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