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According to mitochondrial DNA evidence, *Parascaris equorum* and *Parascaris univalens* may represent the same species

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Abstract

Parascarosis is caused mainly by parasitic infections with *Parascaris equorum* and *Parascaris univalens*, the most common ascarid nematodes, in the small intestine of equines. Parascarosis often causes severe illness and even death in foals and yearlings. In this study, we obtained the complete sequence of the *P. equorum* mitochondrial (mt) genome and compared its organization and structure with that of *P. equorum* Japan isolate (nearly complete), and the complete mtDNA sequences of *P. univalens* Switzerland and USA isolates. The complete mtDNA genome of *P. equorum* China isolate is 13,899 base pairs (bp), making it the smallest of the four genomes. All four *Parascaris* mt genomes are circular, and all genes are transcribed in the same direction. The *P. equorum* mtDNA genome consists of 12 protein-coding genes, two ribosomal RNA genes, 22 transfer (t) RNA genes and one non-coding region, which is consistent with *P. equorum* Japan isolate and *P. univalens* Switzerland isolate but distinct from *P. univalens* USA isolate, which has 20 tRNA genes. Differences in nucleotide sequences of the four entire mt genomes range from 0.1–0.9%, and differences in total amino acid sequences of protein-coding genes are 0.2–2.1%. Phylogenetic analyses showed that the four *Parascaris* species clustered in a clade, indicating that *P. equorum* and *P. univalens* are very closely related. These mt genome datasets provide genetic evidence that *P. equorum* and *P. univalens* may represent the same species, which will be of use in further studies of the taxonomy, systematics and population genetics of ascarids and other nematodes.

Introduction

The Equidae are important reservoir hosts for a variety of nematode parasites, some of which can cause significant morbidity or mortality if their hosts are untreated. Adult horses infected with roundworms present with a variety of clinical symptoms, including nasal discharge, coughing, anorexia, and lethal intestinal obstruction and/or rupture. Moreover, the infection of foals and yearlings can cause severe illness and even death (Morsy *et al.*, 2016).

Parascaris equorum, a large roundworm infecting horses, has been found to occur in many countries, including Sudan, Egypt, Iran, the UK, Australia and China (Beasley *et al.*, 2015; Chang *et al.*, 2015; Easton *et al.*, 2016; Ismail *et al.*, 2016; Morsy *et al.*, 2016; Tavassoli *et al.*, 2016). *Parascaris equorum* is a well-known equine ascarid species but *Parascaris univalens*, which also infects horses, is often overlooked. *Parascaris univalens* is distributed mainly in America and Switzerland (Jabbar *et al.*, 2014; Nielsen *et al.*, 2014). Both species were first described over 130 years ago by Van Beneden (1884). Initially, equine ascarid parasites were considered to be a single species with two substrains, namely *Ascaris megalocephala bivalens* and *A. megalocephala univalens*. Researchers then classified them into separate species, but they are notoriously difficult to distinguish morphologically (Boveri, 1887; Nielsen *et al.*, 2014).

In the early 1980s, cytological techniques distinguished *P. equorum* and *P. univalens* by the number of chromosomes, but this was not verified in a veterinary parasitology study (Jabbar *et al.*, 2014). The mitochondrial (mt) genome has been widely used as a genetic marker in the identification and differentiation of closely related species (Lin *et al.*, 2012; Liu *et al.*, 2012; Gao *et al.*, 2017). However, to date, only 14 mt genomes of horse parasitic nematodes have been reported. Although the nearly complete mtDNA sequence of *P. equorum* Japan isolate has been deposited in the National Center for Biotechnology Information (NCBI) database, obtaining the complete mtDNA sequence is essential to study the population genetics of *Parascaris* in domestic and wild horses globally. Therefore, this study aimed to determine the complete mtDNA sequence of *P. equorum* China isolate and to analyse the genetic relationships among different regional sources of *Parascaris*.

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Table 1. Position and nucleotide sequence length of mitochondrial genomes of *Parascaris equorum* and *Parascaris univalens*, including initiation and termination codons for protein-coding genes and their tRNA gene anticodons (starting from *cox*1).

Genes	Positions and nucleotide sequence lengths (bp)				Number of amino acids				Ini/Ter codons				Anti-codons
	PE	PEJ	PUS	PUU	PE	PEJ	PUS	PUU	PE	PEJ	PUS	PUU	
*cox*1	1–1578 (1578)	1–1578 (1578)	1–1569 (1569)	1–1578 (1578)	525	525	522	525	TTG/TAG	TTG/TAG	TTG/TAG	TTG/TAG	
*trn*C	1579–1633 (55)	1578–1632 (55)	1568–1625 (58)	1578–1632 (55)									GCA
*trn*M	1634–1691 (58)	1633–1693 (61)	1624–1684 (61)	1633–1690 (58)									CAT
*trn*D	1695–1750 (56)	1694–1749 (56)	1686–1740 (55)	1694–1749 (56)									GTC
*trn*G	1754–1809 (56)	1753–1808 (56)	1744–1799 (56)	1753–1808 (56)									TCC
*cox*2	1810–2508 (699)	1809–2507 (699)	1800–2498 (699)	1788–2507 (720)	232	232	232	239	TTG/TAG	TTG/TAG	TTG/TAG	ATC/TAG	
*trn*H	2510–2564 (55)	2509–2563 (55)	2500–2555 (56)	2509–2563 (55)									GTG
*rrn*L	2565–3543 (979)	2564–3524 (961)	2555–3515 (961)	2564–3524 (961)									
*nad*3	3544–3879 (336)	3525–3860 (336)	3515–3850 (336)	3525–3860 (336)	111	111	111	111	TTG/TAG	TTG/TAG	TTG/TAG	TTG/TAG	
*nad*5	3880–5464 (1585)	3861–5445 (1585)	3851–5432 (1582)	3861–5505 (1645)	528	528	527	548	ATT/T	ATT/T	ATT/T	ATT/T	
*trn*A	5465–5520 (56)	5446–5501 (56)	5433–5488 (56)	–									TGC
*trn*P	5522–5577 (57)	5503–5558 (56)	5489–5546 (58)	5506–5561 (56)									TGG
*trn*V	5578–5633 (56)	5559–5614 (56)	5545–5602 (58)	5562–5617 (56)									TAC
*nad*6	5634–6068 (435)	5615–6049 (435)	5602–6036 (435)	5597–6052 (456)	144	144	144	151	ATG/TAG	ATG/TAG	ATG/TAG	ATG/TAG	
*nad*4L	6072–6305 (234)	6053–6286 (234)	6040–6273 (234)	6056–6289 (234)	77	77	77	77	ATT/TAA	ATT/TAA	ATT/TAA	ATT/TAG	
*trn*W	6311–6366 (56)	6292–6347 (56)	6279–6334 (56)	6295–6350 (56)									TCA
*trn*E	6367–6423 (57)	6348–6404 (57)	6335–6391 (57)	6351–6407 (57)									TTC
*rrn*S	6424–7123 (700)	6405–7104 (700)	6392–7093 (702)	6409–7106 (698)									
*trn*S2 (UCN)	7124–7188 (65)	7105–7169 (65)	7092–7156 (65)	7993–8050 (58)									TGA
NCR	7189–7688 (500)	7170–7720 (551)	7157–7704 (548)	7173–8164 (992)									
*trn*N	7689–7747 (59)	7721–7779 (59)	7705–7763 (59)	8144–8202 (59)									GTT
*trn*Y	7769–7825 (57)	7799–7855 (57)	7783–7839 (57)	8222–8277 (56)									GTA
*nad*1	7826–8701 (876)	7856–8728 (873)	7837–8712 (876)	8276–9151 (876)	291	290	291	291	TTG/TAG	TTG/TAG	TTG/TAG	TTG/TAG	
*atp*6	8708–9307 (600)	8735–9332 (598)	8719–9318 (600)	9158–9757 (600)	199	199	199	199	ATT/TAA	ATT/T	ATT/TAA	ATT/TAG	
*trn*K	9306–9367 (62)	9333–9394 (62)	9315–9379 (65)	9756–9817 (62)									TTT
*trn*L2 (UUR)	9376–9430 (55)	9403–9457 (55)	9387–9441 (55)	9826–9880 (55)									TAA
*trn*S1 (AGN)	9431–9484 (54)	9458–9511 (54)	9442–9495 (54)	–									GCT
*nad*2	9485–10,328 (844)	9512–10,355 (844)	9490–10,339 (850)	9905–10,846 (942)	281	281	283	313	TTG/T	TTG/T	TTG/T	ATC/TAA	
*trn*I	10,329–10,387 (59)	10,356–10,414 (59)	10,339–10,399 (61)	10,779–10,837 (59)									GAT
*trn*R	10,387–10,444 (58)	10,415–10,470 (56)	10,398–10,455 (58)	–									ACG
*trn*Q	10,447–10,501 (55)	10,474–10,528 (55)	10,458–10,512 (55)	10,897–10,951 (55)									TTG
*trn*F	10,506–10,562 (57)	10,533–10,590 (58)	10,517–10,574 (58)	10,956–11,013 (58)									GAA

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cytb	10,560–11,666 (1104)	10,588–11,691 (1104)	10,581–11,675 (1095)	10,969–12,114 (1146)	367	367	364	381	GTT/TAG	GTG/TAG	GTT/TAG	ATG/TAG	
trnL1 (CUN)	11,674–11,730 (57)	11,702–11,758 (57)	11,686–11,742 (57)	12,125–12,181 (57)									TAG
cox3	11,731–12,498 (768)	11,759–12,526 (768)	11,743–12,510 (768)	12,200–12,949 (750)	255	255	255	249	GTT/TAG	GTT/TAG	GTT/TAG	ATT/TAG	
trnT	12,497–12,554 (58)	12,526–12,581 (56)	12,509–12,566 (58)	12,949–13,004 (56)									TGT
nad4	12,554–13,783 (1230)	12,582–13,811 (1230)	12,566–13,795 (1230)	12,966–14,234 (1269)	409	409	409	422	TTG/TAA	TTG/TAA	TTG/TAA	ATA/T	
Total	13,899	13,927	13,920	14,350	3419	3418	3414	3506					

PE, Parascaris equorum in this study; PEJ, Parascaris equorum Japan isolate; PUS, Parascaris univalens Switzerland isolate; PUU, Parascaris univalens USA isolate.

Materials and methods

Parasites and extraction of genomic DNA

Adult nematodes of *P. equorum* were obtained from the small intestine of a naturally infected horse in Daqing, Heilongjiang Province, China, and then washed in physiological saline. The nematodes were initially identified at the species level according to primarily morphological characteristics, using existing keys and descriptions (Taylor *et al.*, 2007), then fixed in 70% (v/v) ethanol and stored at –20°C until DNA extraction. Total genomic DNA was isolated from single adult worms using sodium dodecyl sulphate/proteinase K treatment, followed by spin-column purification (Wizard® SV Genomic DNA Purification System, Promega, Madison, Wisconsin, USA). To independently verify the identity of the specimen, the internal transcribed spacer (ITS) of nuclear ribosomal DNA was amplified by polymerase chain reaction (PCR) with the universal primers NC5 (5′-GTAGGTGAACCTG CGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCT CCGCT-3′) and sequenced according to an established method (Gasser *et al.*, 2008). The ITS (accession number: MF678787) sequence obtained was a perfect match with that of *P. equorum* (accession number: JN617987).

PCR amplification, sequence analyses and comparative analyses

The entire mt genome of *P. equorum* was amplified by PCR using 10 primers (supplementary table S1) designed from the conserved regions of *Ascaris suum* (HQ704901) and other Ascarididae nematodes. PCR details have been described in previous studies (Xu *et al.*, 2015; Zhang *et al.*, 2015). Positive PCR products were sequenced at Life Technology Company (Beijing, China) using primers employed in primary amplifications.

Sequences were assembled manually and aligned against the complete mt genome sequences of *A. suum* (HQ704901) using the program Clustal X 1.83 (Thompson *et al.*, 1997) to infer gene boundaries. Twelve protein-coding genes were identified based on comparisons with *A. suum* (HQ704901). The secondary structures of 22 transfer (t) RNA genes were predicted using tRNAscan-SE (Lowe & Eddy, 1997) and/or manual adjustment, and ribosomal (r) RNA genes were identified by comparison with *A. suum* (HQ704901).

The mtDNA size, percentage of A + T content, position and length of the 12 protein-coding genes, identity of the complete mtDNA sequences, and nucleotide and amino acid sequences of the 12 protein-coding genes were compared among the four *Parascaris* species (*P. equorum* China and Japan isolates, *P. univalens* Switzerland and USA isolates).

Phylogenetic analyses

The amino acid sequences conceptually translated from individual genes of the mt genome of *P. equorum* were concatenated. Concatenated amino acid sequences predicted from the published mt genomes of 11 Ascarididae nematodes were selected for phylogenetic analyses: *Ascaris lumbricoides*, NC_016198; *A. suum*, HQ704901; *Baylisascaris procyonis*, NC_016200; *Baylisascaris schroederi*, NC_015927; *Baylisascaris ailuri*, NC_015925; *Baylisascaris transfuga*, NC_015924; *P. univalens* Switzerland isolate, KM067271; *P. univalens* USA isolate, KM216010; *P. equorum* Japan isolate, AP017696; *Toxascaris leonina*, NC_023504; and *Toxocara canis* NC_010690 as the outgroup. All amino acid

Table 2. Identity of nucleotides and predicted amino acids for protein-coding genes in *Parascaris equorum* and *Parascaris univalens*.

Protein-coding genes	Identity of nucleotides/amino acids (%)					
	PE/PEJ	PE/PUS	PE/PUU	PEJ/PUS	PEJ/PUU	PUS/PUU
*cox*1	99.7/99.6	99.7/99.4	99.7/99.6	99.9/99.8	99.9/100.0	100.0/100.0
*cox*2	99.7/100.0	99.9/100.0	99.4/99.6	99.9/100.0	99.4/99.6	99.6/99.6
*nad*3	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0
*nad*5	97.8/95.5	97.9/95.1	97.7/95.6	99.9/99.1	99.7/99.8	99.8/99.2
*nad*6	99.1/98.6	99.1/98.6	98.9/97.9	100.0/100.0	99.8/99.3	99.8/99.3
*nad*4L	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0
*nad*1	99.8/99.0	99.9/99.7	99.5/99.3	99.9/99.3	99.5/99.0	99.7/99.7
*atp*6	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0
*nad*2	99.6/99.6	99.8/99.3	99.8/99.3	99.9/99.6	99.9/99.6	100.0/100.0
*cyt*b	99.9/100.0	99.9/99.7	99.7/99.5	100.0/99.7	99.8/99.5	99.8/99.5
*cox*3	99.1/96.9	99.1/96.9	99.1/96.8	100.0/100.0	100.0/99.6	100.0/99.6
*nad*4	96.3/93.2	96.4/93.2	96.4/92.9	99.9/100.0	99.9/99.8	100.0/100.0
Total	99.1/98.0	99.1/97.9	99.2/97.9	99.8/99.8	99.7/99.7	99.9/99.6

PE, *Parascaris equorum* in this study; PEJ, *Parascaris equorum* Japan isolate; PUS, *Parascaris univalens* Switzerland isolate; PUU, *Parascaris univalens* USA isolate.

sequences (considering all homologous characters) were aligned using MAFFT 7.122 (Katoh & Standley, 2013), and ambiguously aligned regions were excluded using the Gblocks online server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), using the options for less stringent selection (Talavera & Castresana, 2007). Phylogenetic analyses were conducted using two methods: Bayesian inference (BI) and maximum likelihood (ML) (Guindon & Gascuel, 2003; Ronquist & Huelsenbeck, 2003). Selected models and detailed process were as previously described in Gao *et al.* (2017). Phylograms were drawn using Tree View v. 1.65 (Page, 1996).

Results and Discussion

Mitochondrial genome organization

The complete mt genome sequence of *P. equorum* China isolate is 13,899 base pairs (bp) and was deposited in GenBank under accession number MF678786. It contains 12 protein-coding genes (*cox*1–3, *nad*1–6, *nad*4L, *cyt*b and *atp*6), 22 tRNA genes and two rRNA genes (*rrn*S and *rrn*L) (table 1). All genes are transcribed in the same direction. Gene arrangement is the same as for most Ascaridida nematode genes, but is distinct from those of Ascaridiidae and Cucullanidae sequenced to date, e.g. *Ascaridia galli* and *Cucullanus robustus* (Liu *et al.*, 2013; Nielsen *et al.*, 2014). The nucleotide composition of *P. equorum* China isolate is biased towards T bases, while C is the least favoured (T = 48.63%, A = 21.62%, G = 21.59%, C = 8.16%); the A + T content of the mt genome is 70.25%, which is in accordance with mt genomes of other Ascaridida nematodes, such as *A. suum* and *C. robustus* (Park *et al.*, 2011; Liu *et al.*, 2012).

The total length of the 12 protein-coding sequences of *P. equorum* China isolate is 10,289 bp, which encodes 3419 amino acids. Of the 12 protein-coding genes, six (*cox*1, *cox*2, *nad*3, *nad*1, *nad*2 and *nad*4) use TTG as an initiation codon, three (*nad*5, *nad*4L and *atp*6) start with ATT, two (*cyt*b and *cox*3) start with GTT, and *nad*6 starts with ATG. Seven genes (*cox*1, *cox*2, *cox*3, *nad*1, *nad*3, *nad*6 and *cyt*b) use TAG as the termination codon, three (*nad*4L, *atp*6 and *nad*4) end with TAA, and two (*nad*5 and *nad*2) finish with incomplete terminations (T). TTG (start codons) and TAG (stop codons) were most frequently observed, which is consistent with other Ascarididae nematodes (Xie *et al.*, 2011; Liu *et al.*, 2012).

The 22 tRNA genes identified range from 54 bp (*trn*S1^{AGN}) to 65 bp (*trn*S2^{UCN}) in length. Prediction of their putative secondary structures (data not shown) showed that all tRNA genes have a TV-replacement loop instead of the TΨC arm and loop, except for *trn*S1^{AGN} and *trn*S2^{UCN}, which have the DHU loop; this is consistent with other Ascarididae nematodes, such as *T. canis* (Jex *et al.*, 2008) and *A. suum* (Liu *et al.*, 2012). *rrn*S and *rrn*L rRNA genes of *P. equorum* China isolate are 700 bp and 979 bp, respectively. *rrn*L is located between *trn*H and *nad*3, and *rrn*S is located between *trn*E and *trn*S2^{UCN}. Only one non-coding region (NCR), located between *trn*S2^{UCN} and *trn*N, was identified in the *P. equorum* China isolate mt genome; this is consistent with *P. equorum* Japan isolate, *P. univalens* Switzerland isolate and *P. univalens* USA isolate, but differs from most metazoan mtDNA sequences. For example, *Rhigonema thysanophora* has five NCRs (Kim *et al.*, 2014). The A + T content of this region of *P. equorum* China isolate is 80.8%, which is consistent with other Ascarididae nematodes (Liu *et al.*, 2012).

Comparative analyses of the mt genome of P. equorum and P. univalens

The *P. equorum* China isolate mt genome sequence is the smallest of the four *Parascaris* nematodes, being 28 bp shorter than that of *P. equorum* Japan isolate, and 21 bp and 451 bp shorter than *P. univalens* Switzerland isolate and *P. univalens* USA isolate, respectively. The NCR in *P. equorum* China isolate is 500 bp, which is also the smallest of the four *Parascaris* nematodes; *P. equorum* Japan isolate, *P. univalens* Switzerland isolate and *P. univalens* USA isolate have NCRs of 551 bp, 548 bp and 992 bp, respectively (table 1). This indicates that the size of the mt

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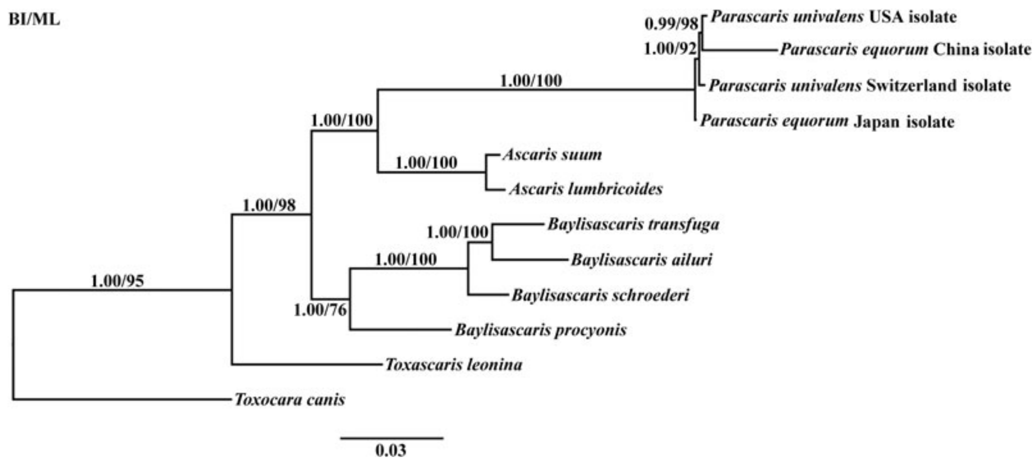

Fig. 1. Phylogenetic relationships among *Parascaris* species and Ascarididae nematodes based on mitochondrial sequence data. The concatenated amino acid sequences of 12 protein-coding genes were analysed by Bayesian inference (BI) and maximum likelihood (ML), using *Toxocara canis* as an outgroup.

genome sequence may correlate with that of the NCR, as previously reported (Gao *et al.*, 2014).

Nucleotide sequences of the entire mt genomes of the four *Parascaris* nematodes differ by 0.1–0.9%, and total amino acid sequences of protein-coding genes differ by 0.2–2.1%. For the 12 protein-coding genes, the magnitude of nucleotide sequence variation ranges from 0 to 3.7%, and amino acid sequence differences range from 0 to 7.1% (table 2), with *nad*3, *nad*4L, and *atp*6 having identical protein sequences, and *nad*4 being the least conserved in this study.

Phylogenetic analyses

To determine the phylogenetic relationship between *P. equorum* and other Ascarididae nematodes, the 12 mtDNA protein-coding genes were analysed using BI and ML methods (fig. 1). The congeneric species (*Ascaris*, *Baylisascaris*, *Parascaris* and *Toxascaris*) formed an independent branch. Nematodes of the genera *Ascaris* and *Parascaris* clustered together, further supporting the findings of Jabbar *et al.* (2014). Importantly, all the *Parascaris* species clustered based on the concatenated amino acid sequences of 12 protein-coding genes. Interestingly, *P. univalens* (Switzerland and USA isolates) and *P. equorum* (Japan and China isolates) were not classified into the same branches. A similar result was reported previously for *A. suum* China isolate and *A. suum* USA isolate (Liu *et al.*, 2012). The clustering of the four *Parascaris* species in a clade with high statistical support in the present study indicates that *P. equorum* and *P. univalens* are very closely related and may even be the same species.

In conclusion, we determined the complete mt genome sequence of *P. equorum* China isolate. Comparative and phylogenetic analyses of mt sequences revealed that *P. equorum* and *P. univalens* may represent the same species. The complete mt genome dataset of *P. equorum* extends what is known about the mt genome of parasitic nematodes.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X18000330

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Conflict of interest. None.

Ethical standards. This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and our protocol was reviewed and approved by the Research Ethics Committee of Heilongjiang Bayi Agricultural University.

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