

# Characterization of the complete mitochondrial genome of *Marshallagia marshalli* and phylogenetic implications for the superfamily Trichostrongyloidea

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Received: 28 August 2017 / Accepted: 30 October 2017 / Published online: 7 November 2017  
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**Abstract** *Marshallagia marshalli* (Nematoda: Trichostrongylidae) infection can lead to serious parasitic gastroenteritis in sheep, goat, and wild ruminant, causing significant socioeconomic losses worldwide. Up to now, the study concerning the molecular biology of *M. marshalli* is limited. Herein, we sequenced the complete mitochondrial (mt) genome of *M. marshalli* and examined its phylogenetic relationship with selected members of the superfamily Trichostrongyloidea using Bayesian inference (BI) based on concatenated mt amino acid sequence datasets. The complete mt genome sequence of *M. marshalli* is 13,891 bp, including 12 protein-coding genes, 22 transfer RNA genes, and 2 ribosomal RNA genes. All protein-coding genes are transcribed in the same direction. Phylogenetic analyses based on concatenated amino acid sequences of the 12 protein-coding genes supported the monophylies of the families Haemonchidae, Molineidae, and Dictyocaulidae with strong statistical

support, but rejected the monophyly of the family Trichostrongylidae. The determination of the complete mt genome sequence of *M. marshalli* provides novel genetic markers for studying the systematics, population genetics, and molecular epidemiology of *M. marshalli* and its congeners.

**Keywords** *Marshallagia marshalli* · Mitochondrial genome · Mitochondrial DNA · Phylogenetic analyses

## Introduction

Gastrointestinal parasites of livestock cause substantial economic losses worldwide (Gasser et al. 2008). *Marshallagia* spp. are among the most common gastrointestinal nematodes of ruminants, and more than 10 species have been recognized in the genus *Marshallagia*. Of them, *Marshallagia marshalli* is widely distributed and is important in tropical and subtropical regions with high prevalence in sheep, goat, and wild ruminants, causing loss of appetite, weight loss, constipation, diarrhea, and even death (Moradpour et al. 2014; Shirvan et al. 2017). It is also one of the most prevalent helminths in ruminants in China, resulting in significant economic losses to the animal industry.

Mitochondrial (mt) genome sequences have been widely used as genetic markers for studying molecular epidemiology, population genetics, and phylogenetics at various taxonomic levels of different organisms (Herd et al. 2015; Song et al. 2016, 2017; Wang et al. 2016; Li et al. 2017), because of their unique characteristics (Wolstenholme 1992; Boore 1999). The current hypothesis of Trichostrongylidae phylogeny was based on morphological and ecological characters and SSU rRNA gene sequence analyses (Hoberg and

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Lichtenfels 1994; Gouÿ de Bellocq et al. 2001). Recently, mt genome sequences have also been used to reconstruct the phylogenetic relationships among Trichostrongylidae nematodes (Jex et al. 2010). Despite these advances, difficulties remain concerning the phylogenetic relationships among Trichostrongylidae nematodes. For example, although some studies (Durette-Desset 1985; Durette-Desset and Chabaud 1993) indicated that Trichostrongylidae is monophyly, others (Gouÿ de Bellocq et al. 2001; Lin et al. 2012; Zhao et al. 2014) have argued the opposite and have suggested that Trichostrongylidae is sister to Haemonchidae and Cooperiidae. These inconsistent hypotheses may result from the inadequate resolution at higher taxonomic levels based on different DNA datasets as well as the uses of different inference methods. In addition, although Trichostrongylidae is a large nematode family, to date, the complete mt genomes of only a limited number of species have been sequenced (Jex et al. 2010). No mt genome has been reported for any of the members of the genus *Marshallagia*. The lack of enough mt genomes of nematodes in this family forms a major limitation for phylogenetic studies of Trichostrongylidae.

Given this background and the significance of *M. marshalli*, the present study aimed to determine the gene content, arrangement, and composition of the mt genome of *M. marshalli* and to reconstruct phylogenetic relationships of the superfamily Trichostrongyloidea using mtDNA sequences.

## Materials and methods

### Parasites and DNA extraction

Two adult specimens of *Marshallagia* were collected from the abomasum of a Tianzhu white yak which was naturally infected in Gansu Province, China. The samples were cleaned by phosphate-buffered saline (PBS) and fixed in 70% ethanol and stored at  $-20\text{ }^{\circ}\text{C}$  until further use. It was difficult to acquire accurate morphological data from the samples preserved in 70% ethyl alcohol; therefore, molecular identification was carried out to determine the identities of the specimens. Total genomic DNA was extracted from *Marshallagia* samples by using sodium dodecyl sulfate (SDS)/proteinase K treatment and spin column purification (Wizard® SV Genomic DNA Purification System, Promega).

### Long-range PCR, sequencing, and annotation

The primers were designed to relatively conserved regions of mt genome sequences of closely related species, namely *Cooperia oncophora* (GQ888713) and *Teladorsagia*

*circumcincta* (GQ888720) (Table 1). The entire mt genome of *M. marshalli* was amplified by long PCR with five overlapping amplicons located between *cox1* and *rrnL* (~ 3.5 kb), *rrnL* and *rrnS* (~ 4.3 kb), *rrnS* and *cytb* (~ 4.1 kb), *cytb* and *cox3* (~ 1.5 kb), and *cox3* and *cox1* (~ 2.4 kb) (Table 1).

Each long-PCR reaction was conducted in a total volume of 50  $\mu\text{l}$ , which included 25  $\mu\text{l}$  PrimeStar Max DNA polymerase premix (Takara, Dalian, China), 25 pmol of each primer (synthesized in Sangon Biotech Company, Shanghai, China), 0.5  $\mu\text{l}$  DNA template, and approximately 24  $\mu\text{l}$   $\text{H}_2\text{O}$ , in a thermocycler (Biometra, Göttingen, Germany). The PCR cycling conditions began with an initial denaturation at  $98\text{ }^{\circ}\text{C}$  for 1.8 min, and then followed with 22 cycles of denaturation at  $98\text{ }^{\circ}\text{C}$  for 18 s, annealing at  $50\text{--}58\text{ }^{\circ}\text{C}$  for 10 s, and extension at  $60\text{ }^{\circ}\text{C}$  for 1.8–5 min, followed by  $98\text{ }^{\circ}\text{C}$  denaturation for 2 min, with additional of 28 cycles of  $98\text{ }^{\circ}\text{C}$  denaturation for 18 s, annealing at  $50\text{--}58\text{ }^{\circ}\text{C}$  for 10 s, and extension at  $60\text{ }^{\circ}\text{C}$  for 1.8–5 min in accordance to the fragment length (average 1000 bp/1 min), with 10 min of the final extension step at  $66\text{ }^{\circ}\text{C}$ . One sample without genomic DNA (negative control) was added in each amplification run. Each amplicon (3  $\mu\text{l}$ ) was detected by electrophoresis in a 0.8% agarose gel and stained with ethidium bromide (Sangon Biotech Company, Shanghai, China) with a clear, single, and bright band. After column purification (Wizard-SV Genomic DNA Purification System, Promega), the products were sequenced by Sangon Biotechnology Company (Shanghai, China) using a primer walking strategy.

The mt genome was annotated using an approach similar to that of Ascaridomorph nematodes and *Gongylonema pulchrum* (Liu et al. 2015; Liu et al. 2016). Briefly, each mt protein-encoding gene was identified by comparison with corresponding genes of the mt genome of a reference species (i.e., *T. circumcincta*, accession number GQ888720) (Jex et al. 2010). The tRNA genes were identified using the program tRNAscan-SE (Lowe and Eddy 1997) or by visual inspection (Hu et al. 2002); rRNA genes were predicted by comparison with those of *T. circumcincta* (Jex et al. 2010).

### Phylogenetic analysis based on concatenated amino acid sequence data

The amino acid sequences conceptually translated from individual genes of the mt genome of *M. marshalli* were concatenated. Concatenated amino acid sequences predicted from published mt genomes of representative nematodes were selected for comparison, including the superfamily Trichostrongyloidea, including the family Haemonchidae (*Haemonchus contortus*, NC\_010383 (Jex et al. 2008); *Haemonchus placei*, NC\_029736; and *Mecistocirrus digitatus*, NC\_013848 (Jex et al. 2010)), the family

**Table 1** Sequences of primers used to amplify PCR fragments of *Marshallagia marshalli*

Primer	Sequence (5' to 3')	Region	Amplicon size (bp)
MAMO1F	GTGGTTTTGGTAATTGAATGTT	<i>cox1-rrnL</i>	~ 3563 bp
MAMO1R	CAATGAATTAACATAATATCACGTT		
MAMO2F	CATTACATTGGGCAACAAAGTTTTCTAG	<i>rrnL-rrnS</i>	~ 4301 bp
MAMO2R	AGCCTAGCCGATTATTCTGGAACAA		
MAMO3F	GTACAAATCATCCATGAATTGCCTA	<i>rrnS-cytb</i>	~ 4119 bp
MAMO3R	ACAACGAACACTAACATACCCAC		
MAMO4F	CGAGGTAAATTATGGTTGAATTTTTCG	<i>cytb-cox3</i>	~ 1578 bp
MAMO4R	CCACACAACACATGAATACCGTGAA		
MAMO5F	ATCAAGGAGAGGGGTCCTGTAACCTT	<i>cox3-cox1</i>	~ 2423 bp
MAMO5R	CGAGGAAACCGTATATCAGGAGCC		

Trichostrongylidae (*Trichostrongylus axei*, NC\_013824; *Trichostrongylus vitrinus*, NC\_013807; *Teladorsagia circumcincta*, NC\_013827) (Jex et al. 2010), the family Cooperiidae (*Cooperia oncophora*, NC\_004806 (Van der Veer and de Vries 2004)), the family Molineidae (*Nematodirus oiratianus*, NC\_024639, and *Nematodirus spathiger*, NC\_024638) (Zhao et al. 2014), and the family Dictyocaulidae (*Dictyocaulus viviparus*, NC\_019810; *Dictyocaulus eckerti*, NC\_019809) (Gasser et al. 2012), using *Oesophagostomum quadrispinulatum* (GenBank accession number NC\_014181) (Lin et al. 2012) as outgroup. Amino acid sequences inferred from the sequences of 12 mt protein-coding genes were aligned individually first using MAFFT 7.122 (Katoh and Standley 2013) and were then concatenated to form a single dataset; ambiguously aligned regions were excluded using Gblocks 0.91b (doc) ([http://molevol.cmima.csic.es/castresana/Gblocks\\_server.html](http://molevol.cmima.csic.es/castresana/Gblocks_server.html)) (Talavera and Castresana 2007) with the default parameters (allow smaller final blocks, allow gap positions within the final blocks, and allow less strict flanking positions). Phylogenetic analysis was conducted using Bayesian inference (BI) as described previously (Liu et al. 2016). Phylograms were drawn using the program FigTree v.1.4 (<http://tree.bio.ed.ac.uk/software/figtree>).

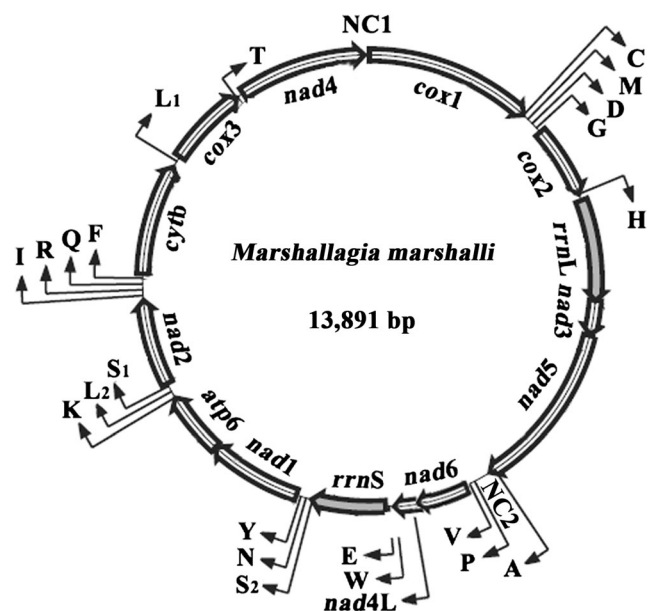
## Results and discussion

### Acquisition of ITS rDNA

A specimen was identified as *M. marshalli* based on PCR-based sequencing of the internal transcribed spacer (ITS-1 and ITS-2) rDNA regions (Newton et al. 1998; Chilton et al. 2001; Nabavi et al. 2014), and both ITS-1 and ITS-2 regions (MG011724) had 99% identity to previously published sequences for *M. marshalli* from Uzbekistan and Iran (GenBank accession nos. KT428384 and HQ389231, respectively).

### Content, organization, and annotation of mt genome

The complete mt genome of *M. marshalli* (GenBank accession no. MG011723) was 13,891 bp in length (Fig. 1), including 12 protein-coding genes (*cox1-3*, *nad1-6*, *nad4L*, *atp6*, and *cytb*), 22 tRNA genes, 2 rRNA genes, and 2 non-coding regions (NCR) (Table 2). The nucleotide composition of the *M. marshalli* mt genome is A = 4131 (29.7%), T = 6458 (46.5%), G = 2308 (16.6%), and C = 994 (7.2%). The gene content and arrangement are the same as those of *H. contortus* (Jex et al. 2008), *T. axei* (Jex et al. 2010), *N. oiratianus* (Zhao et al. 2014), and *D. viviparus* (Gasser et al. 2012).



**Fig. 1** Arrangement of the mitochondrial genome of *M. marshalli*. The scales are similar. All genes are transcribed in the clockwise direction, using standard nomenclature. The 22 tRNA genes are represented by the one-letter code for the corresponding amino acid, with numerals differentiating each of the two leucine-specifying and serine-specifying tRNAs (L1 and L2 for codon families CUN and UUR, respectively; S1 and S2 for codon families AGN and UCN, respectively)

**Table 2** The features of the mitochondrial genome of *Marshallagia marshalli*

Gene/region	Positions and nt sequence sizes (bp)	Amino acid no.	Start/stop codons
<i>cox1</i>	1–1576 (1576)	525	ATT/T
<i>trnC</i>	1579–1634 (56)		
<i>trnM</i>	1682–1737 (56)		
<i>trnD</i>	1739–1794 (56)		
<i>trnG</i>	1800–1855 (56)		
<i>cox2</i>	1856–2551 (696)	231	ATT/TAA
<i>trnH</i>	2551–2604 (54)		
<i>rrnL</i>	2605–3563 (959)		
<i>nad3</i>	3564–3899 (336)	112	ATA/TAA
<i>nad5</i>	3907–5488 (1582)	527	ATT/T
<i>trnA</i>	5489–5545 (57)		
LNCR	5546–5890 (345)		
<i>trnP</i>	5891–5947 (57)		
<i>trnV</i>	5953–6008 (56)		
<i>nad6</i>	6009–6443 (435)	144	TTG/TAA
<i>nad4L</i>	6446–6676 (231)	76	ATG/TAA
<i>trnW</i>	6678–6732 (55)		
<i>trnE</i>	6774–6828 (55)		
<i>rrnS</i>	6835–7530 (696)		
<i>trnS</i>	7531–7583 (53)		
<i>trnN</i>	7627–7681 (55)		
<i>trnY</i>	7733–7787 (55)		
<i>nad1</i>	7791–8663 (873)	290	ATT/TAA
<i>atp6</i>	8659–9258 (600)	199	ATT/TAA
<i>trnK</i>	9259–9318 (60)		
<i>trnL</i>	9386–9441 (56)		
<i>trnS</i>	9442–9493 (52)		
<i>nad2</i>	9494–10,328 (835)	278	TTG/TAG
<i>trnI</i>	10,332–10,390 (59)		
<i>trnR</i>	10,400–10,453 (54)		
<i>trnQ</i>	10,488–10,540 (53)		
<i>trnF</i>	10,545–10,598 (54)		
<i>cytb</i>	10,599–11,710 (1112)	370	ATA/TA
<i>trnW</i>	11,711–11,764 (54)		
<i>cox3</i>	11,768–12,530 (763)	254	ATA/T
<i>trnT</i>	12,531–12,586 (56)		
<i>nad4</i>	12,587–13,813 (1227)	408	ATT/TAA
SNCR	13,814–13,891 (78)		

*M. marshalli* mt genome encoded 12 protein-encoding genes, with 3414 amino acids in total. It has four initiation codons (ATT, ATA, TTG, ATG) and four termination codons (TAA, TAG, TA, T). Among them, ATT is the highest frequency of being used as initiation codons, which has been used six times in total, by *cox1*, *cox2*, *nad5*, *nad1*, *nad4* and *atp6*. ATA with secondary high rate of recurrence (three times) as start codons, *nad3*, *cytb*, and *cox3*, used it in the mt genome of *M. marshalli*. Moreover, TTG are used by *nad6* and *nad2* in the mt genome. As far as stop codons are concerned, TAA is

the most frequently used with seven times altogether, by *cox2*, *nad3*, *nad6*, *nad4L*, *nad1*, *atp6*, and *cox3*. The genes of *nad5*, *cox3*, and *cox1* use T as termination codons. Furthermore, there is abbreviated termination codon TAG being used in *nad2* of the mt genomes, while TA is also another unfinished stop codon being used by *cytb* genes of *M. marshalli*. These results are consistent with the arrangement in the mt genomes of other Trichostrongyloidea nematodes (*Trichostrongylus axei*, *Trichostrongylus vitrinus*, and *Teladorsagia circumcincta*) (Jex et al. 2010).

**Table 3** Nucleotide composition and skew of *Marshallagia marshalli* mitochondrial protein-coding genes

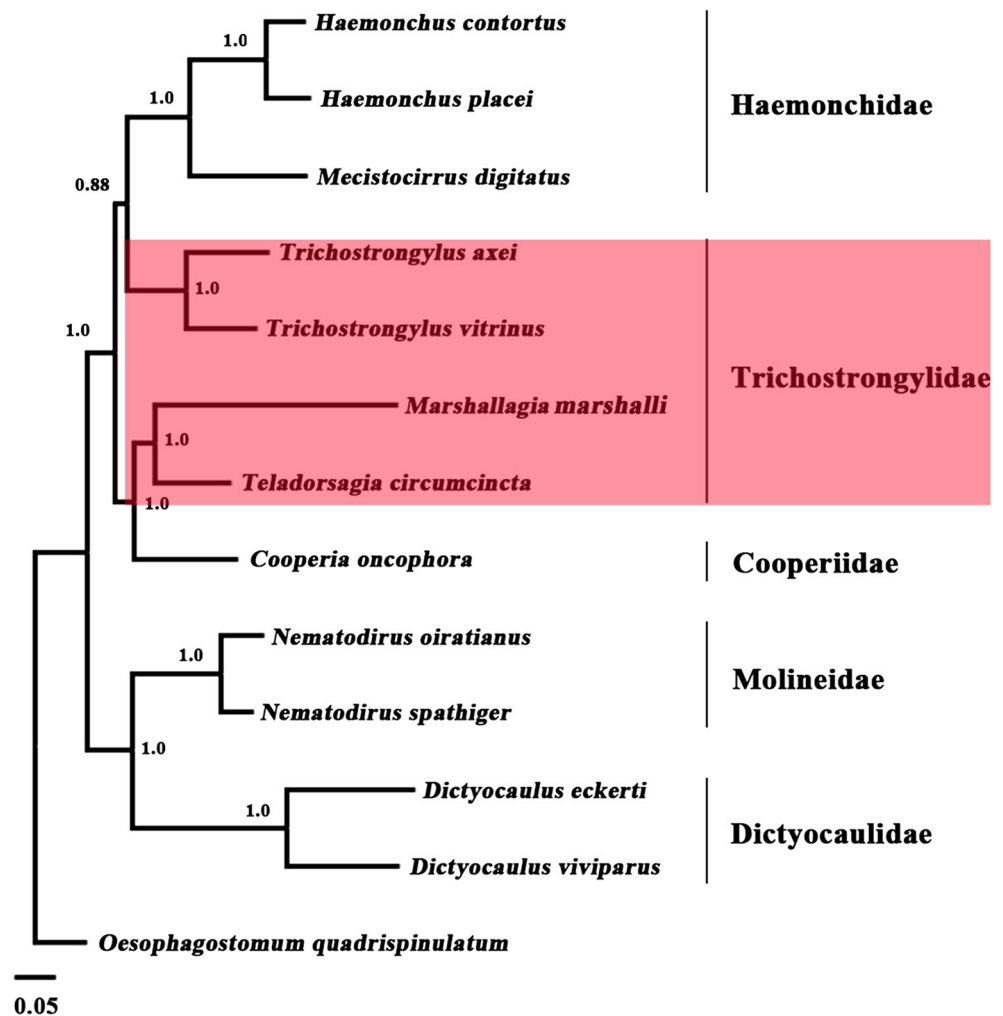
Gene	A	T	G	C	A+T (%)	AT skew	GC skew
<i>cox1</i>	23.5	44.2	22.1	10.3	67.7	-0.31	0.36
<i>cox2</i>	30.5	44.1	17.4	8.0	74.6	-0.18	0.37
<i>nad3</i>	28.6	48.8	18.5	4.1	77.4	-0.26	0.64
<i>nad5</i>	28.3	49.5	15.5	6.7	77.8	-0.27	0.40
<i>nad6</i>	24.4	51.7	19.3	4.6	76.1	-0.36	0.62
<i>nad4L</i>	27.7	55.4	13.9	3.0	83.1	-0.33	0.64
<i>nad1</i>	25.3	46.9	19.9	7.9	72.2	-0.30	0.43
<i>atp6</i>	27.3	48.4	17.8	6.5	75.7	-0.28	0.47
<i>nad2</i>	27.8	50.8	15.5	5.9	78.6	-0.29	0.45
<i>cytb</i>	26.9	46.0	18.6	8.5	72.9	-0.26	0.37
<i>cox3</i>	23.2	47.1	20.3	9.4	70.3	-0.34	0.37
<i>nad4</i>	27.1	50.0	14.9	8.0	77.1	-0.30	0.30
<i>rrnL</i>	39.4	43.2	11.8	5.6	82.6	-0.05	0.35
<i>rrnS</i>	37.5	40.8	14.7	7.0	78.3	-0.04	0.35
LNCR	41.7	48.1	7.0	3.2	89.8		
SNCR	34.6	43.6	9.0	12.8	78.2		
Overall	29.7	46.5	16.6	7.2	76.2	-0.22	0.40

There are 22 tRNA sequences in the mt genome of *M. marshalli* ranging from 51 to 65 nucleotides in length. The *rrnS* gene of *M. marshalli* is located between *trnE* and *trnS* genes and has a length of 696 bp. The *rrnL* gene is located between *trnH/T* and *nad3* genes and is 959 bp in length. Both the *rrnS* and *rrnL* are high in A+T contents, 78.3 and 82.6%, respectively (Table 3). The longer non-coding region (LNCR, 345 bp in length) is located between the *trnA* and *trnP*, and the shorter one (SNCR, 78 bp in size) is located between *nad4* and *cox1* genes (Table 2). The A+T contents of the LNCR is 89.8%, and the SNCR is 78.2% (Table 3). These non-coding regions may be important for the replication and transcription processes, although these actual processes are still unknown (Shadel and Clayton 1997).

### Phylogenetic analysis

The phylogenetic tree was inferred from the concatenated amino acid sequences of 12 key nematodes representing the superfamily Trichostrongyloidea (Fig. 2). Our results

**Fig. 2** Phylogenetic relationships of *M. marshalli* and other Trichostrongyloidea nematodes. Tree inferred from the concatenated amino acid sequence dataset for 12 protein-coding genes from 12 Trichostrongyloidea nematodes was performed by Bayesian inference (BI). *Oesophagostomum quadrispinulatum* (GenBank accession number NC\_014181) was chosen as the outgroup



supported the monophyly of the families Haemonchidae, Molineidae, and Dictyocaulidae with strong statistical support (Bayesian posterior probabilities = 1.0, Fig. 2), but rejected the monophyly of the family Trichostrongylidae, which were consistent with those of previous studies (Gouÿ de Bellocq et al. 2001; Lin et al. 2012; Zhao et al. 2014). Two species from the family Trichostrongylidae were more closely related to *H. contortus*, *H. placei*, and *M. digitatus* (Haemonchidae) than they were to the other two species (*T. circumcincta* and *M. marshalli*) from the family Trichostrongylidae. The close relationship between the species of the family Trichostrongylidae and *H. contortus*, *H. placei*, and *M. digitatus* (Haemonchidae) was moderately supported in BI (Bayesian posterior probabilities = 0.88, Fig. 2). *N. oiratianus* and *N. spathiger* (Molineidae) was more closely related to *D. viviparous* and *D. eckerti* (Dictyocaulidae) than *C. oncophora* (Cooperiidae), *Trichostrongylus* spp., *T. circumcincta* and *M. marshalli* (Trichostrongylidae), and *Haemonchus* spp. and *M. digitatus* (Haemonchidae). These results were consistent with previous study (Zhao et al. 2014).

### Significance and implications

Animal infections with gastrointestinal nematodes (including marshalliasis) can sometimes be diagnosed based on clinical symptoms, such as diarrhea, anemia, mortality, or decreased fertility (Gasser et al. 2008). However, this approach is usually unreliable, because these clinical signs in animals can be caused by one or more members of the gastrointestinal nematodes or other nematodes. Larval stages of *M. marshalli* cannot be identified reliably morphologically. Fortunately, DNA technological approaches have been used as diagnostic methods for many nematodes (Fernández-Soto et al. 2016; Lodh et al. 2016; Roeber et al. 2017; Solórzanogarcía and Pérezponce 2017). Molecular markers, such as the first internal transcribed spacer (ITS-1) region of nuclear rDNA, have been used as alternative tools for clinical diagnosis and molecular epidemiological investigations of *M. marshalli* (Dallas et al. 2000; Dallas et al. 2001). The characterization of the mt genome of *M. marshalli* now provides novel genetic markers for developing new analytical and diagnostic tools.

Mt genome sequences, in particular the protein-coding gene sequences, have been successfully used for examining systematic status of nematodes (Aghazadeh et al. 2015; Blouin 2002; Hawash et al. 2015; Liu et al. 2015; Sun et al. 2016, 2017; Wang et al. 2016; Kim et al. 2017). Therefore, in this study, we determined the mt genome of *M. marshalli* which stimulates a reassessment of the systematic relationships of Trichostrongyloidea nematodes using mt genomic datasets. There have been controversies regarding the

systematics of members of the Trichostrongyloidea (including Haemonchidae, Molineidae, Cooperiidae, Trichostrongylidae, and Dictyocaulidae). To date, mt genomes of many species of the family Trichostrongylidae are still underrepresented or not represented. Therefore, expanding taxa sampling is necessary for future phylogenetic studies of Trichostrongylidae species using mt genomic dataset.

### Conclusion

The present study determined the complete mt genome sequence of *M. marshalli*. Phylogenetic analyses rejected the monophyly of the family Trichostrongylidae. The availability of the *M. marshalli* mt genome sequences provides novel genetic markers for studying the systematics, population genetics, and molecular epidemiology of *M. marshalli* and its congeners.

**Funding Information** This project was supported by the Scientific Research Fund of Hunan Provincial Education Department (Grant No. 16A102), the “Special Fund for Agro-scientific Research in the Public Interest” (Grant No. 201303037), and the Fundamental Research Funds of Chinese Academy of Agricultural Sciences (Grant Nos. Y2016JC05 and 1610312017004).

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