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Morphological and molecular confirmation of the validity of *Trichuris rhinopiptheroxella* in the endangered golden snub-nosed monkey (*Rhinopithecus roxellana*)

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Abstract

The golden snub-nosed monkey (*Rhinopithecus roxellana*) is an endangered species endemic to China. Relatively little is known about the taxonomic status of soil-transmitted helminths (STH) in these monkeys. *Trichuris* spp. (syn. *Trichocephalus*) are among the most important STHs, causing significant socio-economic losses and public health concerns. To date, five *Trichuris* species have been reported in golden monkeys, including a novel species, *T. rhinopiptheroxella,* based on morphology. In the present study, molecular and morphological analysis was conducted on adult *Trichuris* worms obtained from a dead golden snub-nosed monkey, to better understand their taxonomic status. Morphology indicated that the adult *Trichuris* worms were similar to *T. rhinopiptheroxella*. To further ascertain their phylogenetic position, the complete mitochondrial (mt) genome of these worms was sequenced and characterized. The mt genome of *T. rhinopiptheroxella* is 14,186 bp, encoding 37 genes. Phylogenetic analysis based on the concatenated amino acids of 12 protein-coding genes (with the exception of *atp*8) indicated that *T. rhinopiptheroxella* was genetically distinct and exhibited 27.5–27.8% genetic distance between *T. rhinopiptheroxella* and other *Trichuris* spp. Our results support *T. rhinopiptheroxella* as a valid *Trichuris* species and suggest that mt DNA could serve as a marker for future studies on the classification, evolution and molecular epidemiology of *Trichuris* spp. from golden snub-nosed monkeys.

Introduction

The golden snub-nosed monkey (*Rhinopithecus roxellana*) is endemic to China and is ranked as a Class I protected species by both the national government of China and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and has been categorized as Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Yongcheng & Richardson, 2008; Ren *et al*., 2012). The total population of *R. roxellana* in the wild is *c.* 10,000–16,000, mainly distributed in the western part of southern Gansu province, the Qinling Mountains (Shaanxi province), the Shennongjia forestry district (Hubei province), and the Sichuan province of China (Chang *et al*., 2012). In addition to habitat challenges, infectious diseases caused by viruses and gastrointestinal nematodes are also responsible for a sharp decrease in the *R. roxellana* population (Li and Yang, 2015).

Trichuris (syn. *Trichocephalus*) species are common soil-transmitted helminths (STH), which parasitize the digestive tracts of golden monkeys and can cause typhlitis, colitis, chronic dysentery, serious malnutrition and even death. Five *Trichuris* species have been reported in golden monkeys (Li and Yang, 2015), and a *Trichuris* sp. isolated from *R. roxellana* has been named as a novel species, *T. rhinopiptheroxella* sp. nov., based on its distinct morphological features (Zhu *et al*., 2000). However, species nomenclature based on morphology lacks the ability to identify closely related species and larval parasites, is time-consuming and requires expert training (McManus and Bowles, 1996; Zhao *et al*., 2013).

In the last decade, molecular approaches have been widely used for characterization of parasite species (Gasser *et al*., 2008; Wang *et al*., 2011; Zhao *et al*., 2012). Sequence analysis of mitochondrial DNA (mt DNA) has been used to study phylogenetic relationships of *Trichuris* spp. from humans, pigs, sheep, monkeys and other wild animals (Liu *et al*., 2012, 2013, 2014a, b; Hawash *et al*., 2015, 2016; Callejón *et al*., 2017). In particular, sequence analysis of the entire mt genome has been widely used to distinguish closely related parasitic species and correctly identify their taxonomic relationships (Wang *et al*., 2011; Liu *et al*., 2012; Zhao *et al*., 2013, 2016). For example, based on mt genomic

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data, distinct genetic differences were observed in *Trichuris* spp. from humans and pigs (Liu *et al.*, 2012), and the phylogenetic position of *Orientobilharzia turkestanicum* belonging to the genus *Schistosoma* was clarified (Wang *et al.*, 2011; Aldhoun *et al.*, 2012). In the present study, the phylogenetic position of *T. rhinopiptheroxella*, which had previously been characterized only morphologically, was analysed using both morphological and mt genomic data.

Materials and methods

Parasites and isolation of total genomic DNA

Adult specimens of *Trichuris* sp. were collected from the stomach of the dead monkey. The worms were rinsed thoroughly with physiological saline (PBS) to remove host tissue particles and preserved in 75% ethanol prior to DNA extraction. The posterior end of each male worm was excised and placed in lactophenol for further morphological observation. The total genomic DNA was extracted from the mid-body section of each worm using proteinase K treatment and purified directly using a column-purification kit (TIANamp Genomic DNA Kit, TIANGEN, Beijing, China), according to the manufacturer's instructions.

Table 1. Sequences of primers used to amplify PCR fragments from the mt genome of *T. rhinopiptheroxella*.

Primer	mt region amplified	Sequence (5'–3')
Short-PCR		
*cox*2-F	p*cox2*	ACAACACTATGCGACTGAGT
*cox*2-R		CACCACATAGCTCTGCACA
*nad*5-F	p*nad5*	CAAGGATTTTTTTGAGATCTTTTTC
*nad*5-R		TAAACCGAATTGGAGATTTTTGTTT
16S-F	p16S	TTGTAAATCTCCTGCCCAATG
16S-R		CAACATCGAGGTCATAATCA
Long-PCR		
S*nad*5-F		AGGAATGGACTAGGGATCTTACG
S*nad*5-R		CGTGCTTATTGCTTCTAGTTGGG
S16S-F		GTAATCTGACTGTGCAAAGGTAG
S16S-R		TCAACATCGAGGTCATAATCAAC

Polymerase chain reaction (PCR) amplification, sequencing of the mt genome

Initially, three partial fragments (p*cox*2, p*nad*5 and p16S) were amplified using the primers described in table 1, which were designed manually based on conserved regions of mt genes of closely related species. Subsequently, the sequences obtained (p*nad*5 and p16S) were used to design species-specific primer sets for the amplification of the entire mt genome of *Trichuris* spp. from golden monkeys, using three long overlapping PCR fragments (*cox*2-*nad*5, *nad*5-16S, 16S-*cox*2) (table 1). Long-range PCR was performed in a 25 µl mixture containing 2 mM $MgCl_2$, 0.5 mM each of dNTP, 2.5 µl 10 × LA PCR Buffer (Mg^{2+} free), 0.4 µM each of primer, 0.25 µl LA Taq (5 U/µl) (TaKaRa, Dalian, China), and 1 µl of DNA, with the following cycling conditions: 92°C for 2 minutes (initial denaturation), then 92°C for 10 s (denaturation), 45°C for 30 s (annealing), and 60°C for 8 minutes (extension) for nine cycles, followed by 92°C for 10 s, 45°C for 30 s, and 60°C for 9 minutes for 25 cycles, and a final extension at 60°C for 10 minutes. A negative control (no DNA) was also included in each PCR reaction. PCR products (5 µl) were examined by electrophoresis in 1% agarose gel with ethidium bromide at 110 V for 30 minutes. Positive PCR amplicons were purified from the agarose gel using the Universal DNA Purification Kit (TIANGEN, Beijing, China). The purified products were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and then transformed into *Escherichia coli* JM109 competent cells (TaKaRa, Dalian, China) as recommended by the manufacturer to obtain positive recombinant plasmids. At least three positive transformants were sequenced in both directions using a primer walking strategy by the Sangon Company (Shanghai, China).

Sequence analyses and annotation of mitochondrial genome

Gene boundaries, protein-coding genes (PCGs) and two ribosomal RNA genes were annotated based on comparison and alignment with the complete mt genome sequences of *Trichuris trichiura* (Liu *et al.*, 2012; Hawash *et al.*, 2015), *Trichuris* sp. GHL-2013 (Liu *et al.*, 2013) and *Trichuris* spp. TTB1 and TTB2 (Hawash *et al.*, 2015) using Clustal X 1.83 (Thompson *et al.*, 1997). Corresponding amino acid sequences were translated from the 12 PCGs using the invertebrate mitochondrial genetic codes in MEGA 5.0 (Tamura *et al.*, 2011) with default settings. The majority of tRNA genes were identified using tRNAscan-SE (Lowe and Eddy, 1997), together with ARWEN (Laslett and Canbäck, 2008), whereas the remaining tRNA genes were recognized manually based on their capability of folding into the tRNA-like secondary structures and anticodon sequences. The nucleotide composition and frequency of the codon usage were calculated using MEGA 5.0 (Tamura *et al.*, 2011).

Phylogenetic analyses

In order to clarify the phylogenetic relations of *Trichuris* sp. from *R. roxellana* with closely related species, a genetic tree of selected nematodes was reconstructed using the Bayesian inference (BI) method within MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003) based on a concatenated dataset of deduced amino acid sequences of 12 mt genomic PCGs (without the *atp*8 gene), with *Trichinella spiralis* as the outgroup. The relatively conserved regions in each PCG were selected using Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with the options for a less stringent selection (Talavera and Castresana, 2007) and then subjected to subsequent phylogenetic analysis. The BI analyses consisted of 1,000,000 metropolis-coupled Markov chain Monte Carlo (MCMC) generations, with trees sampled every 1000 generations. Bayesian posterior probabilities (PP) were calculated in the remaining trees after discarding the initial 25% of trees as burn-in. Phylograms were shown using the program Tree View 1.65 (Page, 1996).

Results and discussion

Morphological characterization of Trichuris sp. from R. roxellana

To morphologically identify the *Trichuris* sp. from *R. roxellana*, morphological features of 40 male and 40 female worms were

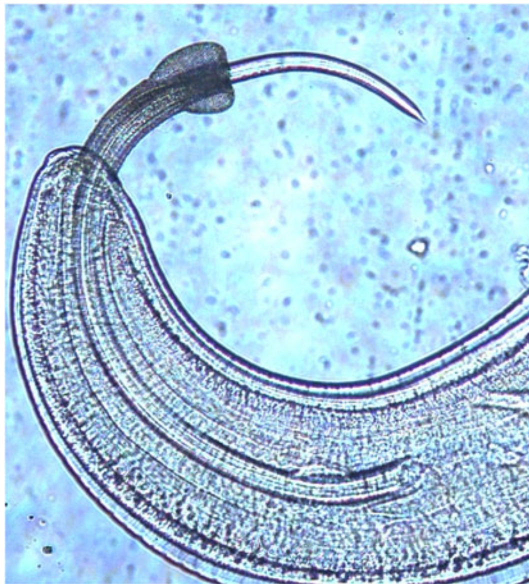

Fig. 1. Posterior end of *T. rhinopiptheroxella* (male), showing the shape of the spicule sheath (40×).

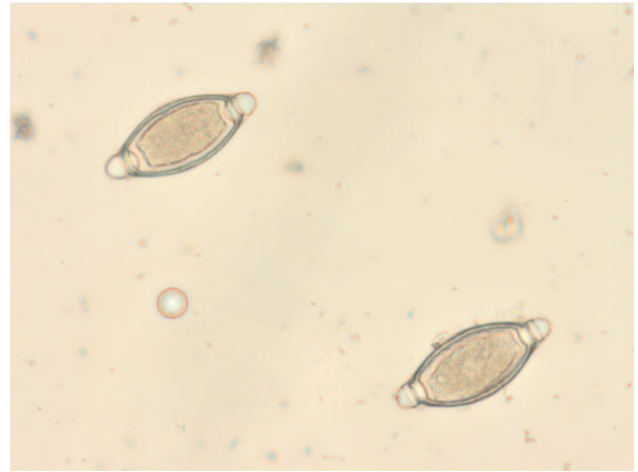

Fig. 2. *Trichuris rhinopiptheroxella* egg (40×).

carefully examined and measured, including the total body length, characters and lengths of the anterior and posterior bodies, inner structures, characters of the spicule and spicule sheath (fig. 1), and eggs (fig. 2). Morphological characterization indicated that the *Trichuris* sp. obtained from a snub-nosed monkey from the Qinling Mountains was similar in measurements to the previously reported *T. rhinopiptheroxella* sp. nov. (Zhu *et al.*, 2000), suggesting that they were the same species of nematode. However, considering that the location of the monkeys differed, we only tentatively identified the nematodes obtained in the present study as *T. rhinopiptheroxella*.

The mitochondrial genome of T. rhinopiptheroxella

The complete mitochondrial genome of *T. rhinopiptheroxella* (GenBank accession number: MG189593) is a typical circular duplex DNA molecule, with a total length of 14,186 bp (fig. 3), which is 140 bp longer than *T. trichiura* from humans (14,046 bp, NC_017750 and GU385218), 39 bp longer than a *Trichuris* sp. GHL-2013 from Francois' leaf-monkey (*Trachypithecus francoisi*) (14,147 bp, KC461179), 202 bp longer than a *Trichuris* sp. TTB1 from the hamadryas baboon (*Papio hamadryas*) (13,984 bp, KT449824), and 177 bp longer than a *Trichuris* sp. TTB2 from an olive baboon (*Papio anubis*) (14,009 bp, KT449825). It contains 13 protein-coding genes (*cox*1-3, *nad*1-6, *nad*4L, *atp*6, *atp*8 and *cyt*b), 22 transfer RNA genes, two ribosomal RNA genes and two non-coding regions (table 2). Moreover, four PCGs (*nad*2, *nad*5, *nad*4 and *nad*4L) and 10 tRNA genes (tRNA-Met, tRNA-Phe, tRNA-His, tRNA-Arg, tRNA-Pro, tRNA-Trp, tRNA-Ile, tRNA-Gly, tRNA-Cys and tRNA-Tyr) are transcribed on the L-strand, whereas the other genes are encoded on the H-strand, which is consistent with mt genomes of other *Trichuris* species previously reported (Liu *et al.*, 2012, 2013; Hawash *et al.*, 2015).

The mt genome sequence of *T. rhinopiptheroxella* has a marked A + T bias, with an overall A + T nucleotide composition of 69.5%, in which T is predominant and G is the least popular nucleotide, in line with the mt genomes of other whipworms (Liu *et al.*, 2012, 2013; Hawash *et al.*, 2015).

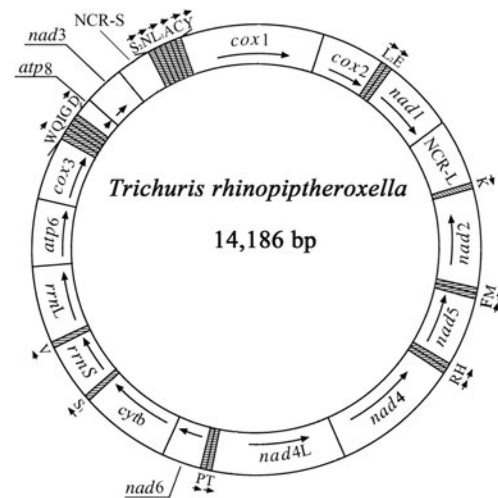

Fig. 3. Circular mapping of the mitochondrial genome of *T. rhinopiptheroxella*. Gene scaling is approximate and all genes are coded by the same DNA strand. The arrow indicates the direction of transcription and all genes have standard nomenclature except for the 22 tRNA genes, which are designated by the one-letter code for the corresponding amino acid. Numerals differentiate each of the two leucine- and serine-specifying tRNA (L1 for codon families CUN and L2 for UUR, S1 for AGN and S2 for UCN) with small coding (NCR) and large non-coding (NCL) regions.

For all 13 PCGs of *T. rhinopiptheroxella*, the ATN codons are commonly used as the start codon, with four (*cox*1-3, *cytb*) using ATG, four (*nad*1, *nad*2, *nad*5 and *nad*6) employing ATA, and two (*nad*3 and *nad*4) employing ATT (table 2). Although most PCGs used TAA (*cox*1-3, *nad*2-6) as the termination codon, an incomplete TA and T were also found to encode termination codons for *atp*6 and *atp*8, respectively, which was not in accordance with studies of the other congeneric nematodes (Liu *et al.*, 2012; Hawash *et al.*, 2015). Excluding the termination codons, a total of 3,577 amino acids (table 3) were encoded by the entire mt genomes of *T. rhinopiptheroxella*. The nucleotide bias towards AT also corresponded with a higher frequency of T-rich codons: ATA for methionine (6.91%), TTT for phenylalanine (5.73%), TTA for leucine (5.59%), and ATT for isoleucine (5.93%). The codons CGG (leucine) were utilized only twice.

All 22 typical tRNA genes were detected in the mt genome of *T. rhinopiptheroxella*, ranging from 51 to 68 bp in size. The

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Table 2. Mitochondrial genome organization in *T. rhinopiptheroxella*.

Gene/Region	Positions	Size (bp)	Strand	Ini/Ter codons
*cox*1	1–1545	1545	H	ATG/TAA
*cox*2	1552–2226	675	H	ATG/TAA
tRNA-LeuUUR (L2)	2241–2303	63	H	
tRNA-Glu (E)	2316–2373	58	H	
*nad*1	2389–3288	900	H	ATA/TAG
Non-coding region (NCL)	3289–3496	208	H	
tRNA-Lys (K)	3497–3560	64	H	
*nad*2	3565–4464	900	L	ATA/TAA
tRNA-Met (M)	4465–4525	61	L	
tRNA-Phe (F)	4520–4581	62	L	
*nad*5	4588–6135	1548	L	ATA/TAA
tRNA-His (H)	6123–6186	64	L	
tRNA-Arg (R)	6186–6249	64	L	
*nad*4	6251–7462	1212	L	ATT/TAA
*nad*4L	7481–7744	264	L	ACA/TAG
tRNA-Thr (T)	7749–7803	55	H	
tRNA-Pro (P)	7805–7858	54	L	
*nad*6	7860–8327	468	H	ATA/TAA
*Cyt*b	8342–9448	1107	H	ATG/TAG
tRNA-SerAGN (S1)	9447–9497	51	H	
rrnS	9497–10196	700	H	
tRNA-Val (V)	10199–10255	57	H	
*rrn*L	10262–11258	997	H	
*atp*6	11235–12064	830	H	CTA/TA
*cox*3	12053–12826	774	H	ATG/TAA
tRNA-Trp (W)	12831–12898	68	L	
tRNA-Gln (Q)	12910–12965	56	H	
tRNA-Ile (I)	12964–13028	65	L	
tRNA-Gly (G)	13035–13091	57	L	
tRNA-Asp (D)	13099–13154	56	H	
*atp*8	13142–13313	168	H	GAT/T
*nad*3	13315–13656	342	H	ATT/TAA
Non-coding region (NCR)	13657–13760	104	H	
tRNA-SerUCN (S2)	13761–13813	54	H	
tRNA-Asn (N)	13813–13870	58	H	
tRNA-LeuCUN (L1)	13880–13947	68	H	
tRNA-Ala (A)	13949–14007	59	H	
tRNA-Cys (C)	14046–14105	60	L	
tRNA-Tyr (Y)	14110–14169	60	L	

putative secondary structures of most tRNA genes (data not shown) shared common features, including an amino-acyl stem, a dihydrouridine (DHU) arm, and an anticodon stem, but lacking the TΨC arm which took the place of a TV replacement loop, with the exception of trnS1 and trnS2, which had a DHU arm that possessed a TΨC stem–loop structure. These structures were similar to those reported in other *Trichuris* spp. (Liu *et al.*, 2012, 2013; Hawash *et al.*, 2015).

The small- (*rrn*S) and large-subunit ribosomal RNA (*rrn*L) of *T. rhinopiptheroxella* were identified based on comparisons with sequences from *T. trichuria* (Liu *et al.*, 2012, 2013; Hawash *et al.*, 2015). *rrn*L and *rrn*S were 997 bp and 700 bp in length, with A + T contents of 74.2% and 74%, respectively, which were located between tRNA-Val and *atp*6, and between tRNA-SerAGN (S1) and tRNA-Val, respectively.

Additionally, two non-coding regions (NCRs) were identified in the mtDNA sequences of *T. rhinopiptheroxella*, including the large (NCL) and small non-coding (NCR) regions. The NCL (208 bp), located between *nad*1 and tRNA-Lys, contained 62 tandem repeats (AT), with the A + T content of 87.5%, while the NCR (109 bp) was situated between *nad*3 and tRNA-SerUCN (S2), with the A + T content of 75.23%. These regions, also referred to as control regions, are known to be likely candidates for replication and transcription (Wolstenholme *et al.*, 1992).

Comparative analyses among monkey- and human-derived Trichuris *sp.*

In the present study, the complete mt genome of *T. rhinopiptheroxella* was sequenced. Genetic distances between *T. rhinopiptheroxella* and other *Trichuris* from humans, Francois' leaf-monkey, the hamadryas baboon and the olive baboon were 27.5%, 27.7%, 27.8% and 27.8%, respectively. The mt gene arrangement of *T. rhinopiptheroxella* was completely identical to that of other congeneric whipworms available in GenBank. In the phylogenetic tree, *T. rhinopiptheroxella* was genetically distinct and clustered in a clade with *Trichuris* sp. GHL-2013 from Francois' leaf-monkey. Previous studies have reported that mt DNA sequence variation between individuals within a species could reach up to 2% on average, whilst between closely related species genetic distances were generally 10–20% (Blouin, 2002). In the present study, the genetic distance of 27.5–27.8% between *T. rhinopiptheroxella* and other *Trichuris* sp. provides further evidence that *T. rhinopiptheroxella* represents a separate species at the molecular level.

Phylogenetic analysis of Trichuris *spp.*

The BI tree based on the concatenated amino acid sequences of 12 PCGs (with the exception of *atp*8) (fig. 4) also confirmed the genetic distinctness of *T. rhinopiptheroxella* from the other *Trichuris* species, with absolute nodal support (pp = 1.00), confirming that *T. rhinopiptheroxella* is a valid species, which is most closely related to *Trichuris* sp. GHL-2013 from Francois' leaf-monkey.

Trichuris, an STH of medical and veterinary importance, can parasitize a broad range of hosts, such as ruminants, humans,

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Table 3. Codon usage of *T. rhinopiptheroxella* mitochondrial DNA encoded proteins.

Amino acid	Codon	Number	Frequency (%)	Amino acid	Codon	Number	Frequency (%)
Phe	TTT	205	5.73	Tyr	TAT	88	2.46
Phe	TTC	93	2.60	Tyr	TAC	78	2.18
Leu	TTA	200	5.59	Term	TAA	9	0.25
Leu	TTG	44	1.23	Term	TAG	3	0.08
Leu	CTT	108	3.02	His	CAT	34	0.95
Leu	CTC	55	1.54	His	CAC	29	0.81
Leu	CTA	146	4.08	Gln	CAA	41	1.15
Leu	CTG	34	0.95	Gln	CAG	6	0.17
Ile	ATT	196	5.48	Asn	AAT	112	3.13
Ile	ATC	92	2.57	Asn	AAC	85	2.38
Met	ATA	247	6.91	Lys	AAA	107	2.99
Met	ATG	48	1.34	Lys	AAG	11	0.31
Val	GTT	54	1.51	Asp	GAT	28	0.78
Val	GTC	26	0.73	Asp	GAC	22	0.62
Val	GTA	63	1.76	Glu	GAA	62	1.73
Val	GTG	11	0.31	Glu	GAG	12	0.34
Ser	TCT	75	2.10	Cys	TGT	28	0.78
Ser	TCC	52	1.45	Cys	TGC	20	0.56
Ser	TCA	62	1.73	Trp	TGA	103	2.88
Ser	TCG	8	0.22	Trp	TGG	21	0.59
Pro	CCT	61	1.71	Arg	CGT	13	0.36
Pro	CCC	32	0.89	Arg	CGC	6	0.17
Pro	CCA	35	0.98	Arg	CGA	22	0.62
Pro	CCG	4	0.11	Arg	CGG	2	0.06
Thr	ACT	102	2.85	Ser	AGT	39	1.09
Thr	ACC	53	1.48	Ser	AGC	31	0.87
Thr	ACA	78	2.18	Ser	AGA	75	2.10
Thr	ACG	7	0.20	Ser	AGG	12	0.34
Ala	GCT	52	1.45	Gly	GGT	36	1.01
Ala	GCC	26	0.73	Gly	GGC	26	0.73
Ala	GCA	53	1.48	Gly	GGA	61	1.71
Ala	GCG	7	0.20	Gly	GGG	26	0.73

Total number of codons for *T. rhinopiptheroxella* is 3577, excluding the incomplete termination codons.

non-human primates, pigs, dogs and rodents (Cafrune *et al.*, 1999; Robles *et al.*, 2014). *Trichuris trichiura* has been reported to infect 600 million people globally (Hotez *et al.*, 2009), especially children, resulting in considerable socio-economic losses and public health concerns. *Trichuris* derived from *R. roxellana* was initially reported in the stomach of golden snub-nosed monkeys and named as *T. rhinopiptheroxella* on the basis of morphology alone (Zhu *et al.*, 2000). However, relying solely on conventional methods may cause taxonomic problems (synonyms) in morphologically similar species (Cutillas *et al.*, 1995; Oliveros *et al.*, 2000). With the advent of molecular biology, mtDNA has been proven as an alternative way to address taxonomic issues of parasites, especially using amino acid datasets inferred from the mt genomes, which have been shown to be robust genetic markers for elucidating phylogenetic relationships at different taxonomic levels (Lü *et al.*, 2010; Liu *et al.*, 2012; Zhao *et al.*, 2012, 2016).

Previous studies reported that the prevalence of *Trichuris* sp. in golden snub-nosed monkeys in Shaanxi province was 83.3%, indicating that this is a common infection in these monkeys (Ravasi *et al.*, 2012). The present study sequenced and characterized the complete mitochondrial genome sequences of *T. rhinopiptheroxella*, further supporting that *T. rhinopiptheroxella* represents a valid *Trichuris* species, and provides a solid basis for further taxonomic, population genetic and molecular epidemiological studies of *Trichuris*.

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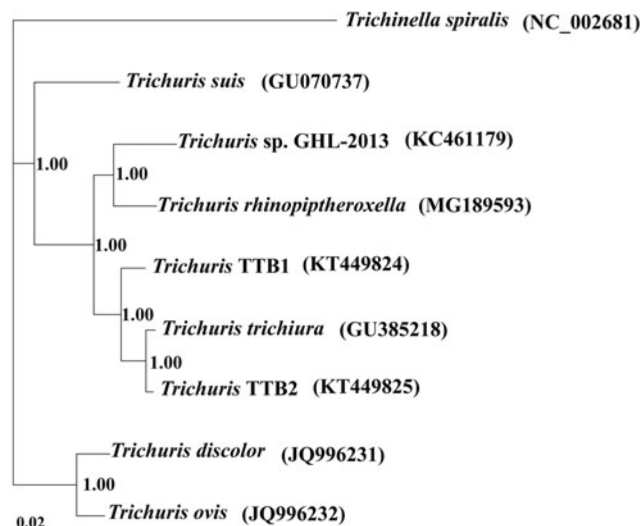

Fig. 4. Genetic relationship of *T. rhinopiptheroxella* with closely related nematode species based on mitochondrial sequence data. The concatenated amino acid sequences of 12 protein-coding genes (with the exception of *atp*8) were analysed using Bayesian inference, with *Trichinella spiralis* as the outgroup; posterior probability (scale bar).

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Conflict of interest. None.

Ethical standards. This study was carried out strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and the protocol was reviewed and approved by the Research Ethics Committee of Northwest A&F University. The golden snub-nosed monkey used in the study was rescued from the Qinling Mountains (34°04′6.29″N, 108°19′19.74″E), but unfortunately died two days later due to age and parasitic infections. Sampling of parasites from the dead monkey was permitted by the Shaanxi Rare Wildlife Rescue Breeding Research Center, with no specific permits being required by the authority for the collection of worms.

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