Mitochondrial and Nuclear Ribosomal DNA Evidence Supports the Existence of a New *Trichuris* Species in the Endangered François’ Leaf-Monkey

Guo-Hua Liu1,4, Robin B. Gasser2*, Peter Nejsum3, Yan Wang1, Qiang Chen5, Hui-Qun Song1, Xing-Quan Zhu1,4*

1 State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province, People’s Republic of China, 2 Faculty of Veterinary Science, The University of Melbourne, Melbourne, Victoria, Australia, 3 Departments of Veterinary Disease Biology and Basic Animal and Veterinary Science, University of Copenhagen, Copenhagen, Denmark, 4 College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan Province, People’s Republic of China, 5 Guangzhou ZhongDa Medical Equipment Co., Ltd., Guangzhou, Guangdong Province, People’s Republic of China

Abstract

The whipworm of humans, *Trichuris trichiura*, is responsible for a neglected tropical disease (NTD) of major importance in tropical and subtropical countries of the world. Whipworms also infect animal hosts, including pigs, dogs and non-human primates, cause clinical disease (trichuriasis) similar to that of humans. Although *Trichuris* species are usually considered to be host specific, it is not clear whether non-human primates are infected with *T. trichiura* or other species. In the present study, we sequenced the complete mitochondrial (mt) genome as well as the first and second internal transcribed spacers (ITS-1 and ITS-2) of *Trichuris* from the François’ leaf-monkey (langur), and compared them with homologous sequences from human- and pig-derived *Trichuris*. In addition, sequence comparison of a conserved mt ribosomal gene among multiple individual whipworms revealed substantial nucleotide differences among these three host species but limited sequence variation within each of them. The molecular data indicate that the monkey-derived whipworm is a separate species from that of humans. Future work should focus on detailed population genetic and morphological studies (by electron microscopy) of whipworms from various non-humans primates and humans.

Citation: Liu G-H, Gasser RB, Nejsum P, Wang Y, Chen Q, et al. (2013) Mitochondrial and Nuclear Ribosomal DNA Evidence Supports the Existence of a New *Trichuris* Species in the Endangered François’ Leaf-Monkey. PLoS ONE 8(6): e66249. doi:10.1371/journal.pone.0066249

Editor: Axel Janke, BfK-Biodiversity and Climate Research Center, Germany

Received January 21, 2013; Accepted May 3, 2013; Published June 20, 2013

Copyright: © 2013 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the International Science & Technology Cooperation Program of China (Grant No. 2013DFA31840) to XQZ and RBG. This work was also supported by the Science Fund for Creative Research Groups of Gansu Province (Grant No. 1210RJA006) to XQZ. RBG’s research is supported by the Australian Research Council (ARC), National Health and Medical Research Council (NHMRC) and Melbourne Water Corporation (MWCl); the Alexander von Humboldt Foundation is also gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Co-author Qiang Chen is an employee of Guangzhou ZhongDa Medical Equipment Co., Ltd. The other authors declare that they have no competing interests.

* E-mail: robinbg@unimelb.edu.au (RBG); xingquanzhu1@hotmail.com (XQZ)

Introduction

Neglected tropical diseases (NTDs) have a devastating effect on animal and human health and food production globally. For instance, it is estimated that more than two billion people are infected with *Ascaris* (common roundworm), *Necator, Ancylostoma* (hookworms) and *Trichuris* (whipworm), mainly in underprivileged areas of the world [1]. *Trichuris trichiura* is a very common parasite of humans in developing countries, and causes trichuriasis in ~600 million people worldwide, mainly in children aged between 5 and 15 years [2]. Trichuriasis can be associated with intestinal symptoms, such as abdominal pain, dysentery, nausea, vomiting, anorexia, constipation and chronic appendiceal syndrome [2]. Whipworms also infect a broad range of other hosts, including pigs (*T. suis*), dogs (*T. vulpis*), sheep (*T. ovis*), goats (*T. skrjabini*), rats (*T. marsi*) and non-human primates, and can cause clinical disease similar to trichuriasis of humans [3–7].

*Trichuris* infects non-human primates in many countries, including Belgium [7], China [8], Ethiopia [9], Kenya [10,11], Peru [12], South Africa [13]. In spite of the high prevalence of *Trichuris* sometimes reported in non-human primates [13], it is not clear whether the non-human primates harbour *T. trichiura* or other congeners. Based on morphological features of adult worms, *Trichuris* of non-human primates (including *Trichuris cynocephalus* and *T. rhinophila*) have been regarded as *T. trichiura* [14,15]. However, the identification of *Trichuris* to species using morphological criteria alone is not reliable. Moreover, neither larval or egg stages of *Trichuris* from humans, pigs and non-human primates can be identified or differentiated unequivocally to species using classical diagnostic approaches [14,16]. Therefore, there is a need for suitable molecular approaches to accurately identify and distinguish closely-related *Trichuris* species from different hosts.

Molecular tools, using genetic markers in mitochondrial (mt) DNA and in the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA), have been used effectively to
identify nematode species [17–21]. For whipworms, mtDNA has been used in China to show clear genetic distinctiveness between human- and pig-derived Trichuris [22], and between T. suis and T. dissolcor from ruminant hosts [23]. Using ITS rDNA, recent studies of Trichuris specimens obtained from humans and pigs [24,25] also indicate that T. trichiura and T. suis are separate species. Cullias et al. [26] used the ITS rDNA to infer the existence of two separate Trichuris species in murid and arvicolid rodents. In other studies from Spain, ITS rDNA has also been employed to distinguish among T. suis from swine, T. vulpis from dogs [27] and T. trichiura from the non-human primates (i.e. Pan troglodytes, Colobus guereza kikyanus and Nomascus gabriellae) [15]. Although a recent investigation has shown two distinct Trichuris genotypes infecting both humans and non-human primates [28], there is still a paucity of information on Trichuris from different species of primates and countries around the world. Therefore, in the present study, we characterized the mt genomic and ITS rDNA sequences of Trichuris from the endangered Francois' leaf-monkey (Presbytis françoisii), which usually lives in close proximity to human populations in southern China [29], and we compared them with homologous sequences of human- and pig-derived Trichuris, and then tested the hypothesis that this monkey-derived Trichuris is a separate species.

Materials and Methods

Ethics Statement

This study did not require approval by an ethics committee. Two Francois’ leaf-monkeys, from which Trichuris specimens were collected from their caeca post-mortem, were handled and housed in a zoo in strict accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People’s Republic of China. The monkeys were caged, and there were two rooms in a cage; one was indoor and the other was outdoor. They were fed fruits and vegetables. The monkeys were under the care and treatment of a licensed veterinarian at the zoo, and were euthanized due to acute gastric dilatation.

Parasites and Isolation of Total Genomic DNA

Two adult specimens of Trichuris (designated “monkey-Trichuris”) were collected from each of the two Francois’ leaf-monkeys, and were washed in physiological saline, identified morphologically [14], fixed in 70% (v/v) ethanol and stored at –20°C until use. Total genomic DNA was isolated separately from four individual worms (coded TH1-TH4) using an established method [30].

Long-range PCR-based Sequencing of mt DNA

To obtain some mt sequence data for primer design, we PCR-amplified regions (400–500 bp) of the cox1 gene by using a (relatively) conserved primer pair JB3 JB4.5 [31], and nad5 gene was amplified using primers NAD5F (forward; 5’- GAACCTGCGGAAGGATTTTTGGTTT-3’ and NAD5R (reverse; 5’- TAAACCGAATTGGAGATTCTTTTTC-3’) described previously [36]. The primers mLFL (5’-AAACCTCGGAAATCGGATAGTAATGTAAT-3’) and mLLR (5’- CGGCCACAGAGCATTGAATTGAAG-3’) designed to conserved mt genome sequences within the mtL gene were employed for PCR amplification and subsequent sequencing of a portion (~600 bp) of this gene from multiple individuals of monkey-derived Trichuris.

Sequence Analyses

Sequences were assembled manually and aligned against the complete mt genome sequences of T. trichiura [22] using the computer program Clustal X 1.83 [33] to infer gene boundaries. The open reading frames (ORFs) were identified using ORFfinder (http://www.ncbi.nlm.nih.gov/orffinder) employing the invertebrate mitochondrial code, and subsequently compared with that of T. trichiura [22]. Translation initiation and termination codons were identified based on comparison with those reported previously [22]. The secondary structures of 22 tRNA genes were predicted using tRNAscan-SE [34] and/or manual adjustment [35], and rRNA genes were identified by comparison with those known for Trichuris [22].

Sequencing of ITS rDNA and mt rrnL

The full ITS rDNA region including primer flanking 18S and 28S rDNA sequences was PCR-amplified from individual DNA samples using universal primers NC5 (forward; 5’-GTAGGT-GAACCTGGAGAAGGATCATTT-3’) and NC2 (reverse, 5’- TTAGCTTTTTTCTCCGGCT-3’) common among all of the nematodes included here were concatenated into a single alignment, and then aligned with those of four other enoplid nematodes (GenBank accession nos. GU385218, GU070737, JQ996232 and JQ996231 for T. trichiura, T. suis, T. ovis and T. dissolcor, respectively), using T. spiralis (accession no. NC_002681) as an outgroup. Ambiguous sites and regions in the alignment were excluded using Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) [38] using default parameters. The rrnL sequences determined here and those of human- and pig-derived Trichuris [22] were aligned and subjected to phylogenetic analysis using Trichinella spiralis (accession

| Table 1. Sequences of primers used to amplify mitochondrial DNA regions from monkey-Trichuris. |
|---------------------------------------------------------------|
| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| THCO1F | GITTCTTCTGCGGACGATTACCTA |
| THND5R | TACTTGTAGTGGCAGGGTTATC |
| THND5F | TAGGAGCCAGCCATAGGATGTAAG |
| TH165R | AATCCGAGAAGTCCGATGGAAG |
| TH165F | TAACAGGCAGAGACCTTAAAGCTT |
| THC01R | CGAAAGATGGATGAGTGACCAAT |
| doi:10.1371/journal.pone.0066249.t001 |
no. NC_002681) [37] as an outgroup (cf. [39]). Phylogenetic analyses were conducted using Bayesian inference [40]; as described previously [40]. Phylogenograms were drawn using the program Tree View v.1.65 [41].

Results

Features of the Circular mt Genome of *Trichuris* from the François Leaf-monkey

The complete mt genome sequence was 14,147 bp in length (GenBank accession no. KC461179). The mt genome contains 13 protein-coding genes (nox-3, nad1-6, nad4L, cytb, atp6 and atp8), 22 transfer RNA genes and two ribosomal RNA genes (rrnL and rrnS) (Table 2); the atp8 gene is encoded (Figure 1). The protein-coding genes are transcribed in different directions, as reported for *T. trichura* and *T. suis* [22] (Table 2). Protein-coding genes were annotated by aligning sequences, and identifying translation initiation and termination codons by comparison with homologous sequences for other whipworms (Table 2).

Twenty-two tRNA genes, which varied from 52 to 67 bp in length, were predicted from the mt genomes. The two ribosomal RNA genes (rrnL and rrnS) were inferred; rrnL is located between tRNA-Val and atp6, and rrnS is located between tRNA-Ser (AGN) and tRNA-Val. The lengths of rrnL and rrnS are 1,007 bp and 705 bp, respectively. The A+T contents of rrnL and rrnS are 69.02% and 70.21%, respectively.

Two AT-rich non-coding regions (NCRs) were inferred in the mt genome. For this genome, the long NCR (designated NCR-L; 124 bp in length) is located between the nad1 and tRNA-Lys (Figure 1), has an A+T content of 59.68%. The short NCR (NCR-S; 105 bp in length) is located between genes nad3 and tRNA-Ser (UCN) (Figure 1), with an A+T content of 79.25%.

Nuclear Ribosomal DNA Regions of *Trichuris* from the Monkey

The rDNA region including ITS-1, ITS-2 and intervening 5.8 rRNA gene sequenced from individual *Trichuris* samples (coded TH1-TH4) was 1,314 bp in length. Individual spacers were 570 bp (ITS-1) and 468 bp (ITS-2), and the 5.8S rRNA gene was 154 bp long.

Comparative Analyses Among Monkey- *Trichuris*, Human-*Trichuris* and Pig-*Trichuris*

The mt genome sequence of monkey-*Trichuris* (accession no. KC461179) was 14,147 bp in length, 101 bp longer than that of human-*Trichuris*, and 289 bp shorter than that of pig-*Trichuris*. The arrangement of the mt genes (i.e., 13 protein genes, 2 rrn genes and 22 tRNA genes) and NCRs were the same. A pairwise comparison of the nucleotide sequences of each mt gene and the amino acid sequences conceptually translated from individual protein genes was made among the three taxa of *Trichuris* (from the three host species) (Table 3). The sequence lengths of individual genes varied among these taxa, except for the nad1 gene, which was the same (Table 3). The magnitude of sequence variation in each gene among the three taxa of *Trichuris* ranged from 24.2–50.9% for nucleotide sequences and 13.6–62.5% for amino acid sequences (Table 3). The sequence difference across the entire mt genome between monkey- and human-*Trichuris* was 29.35% (a total of 4,152 nucleotide alterations). This difference across the entire mt genome between monkey- and pig-*Trichuris* was 33.49% (a total of 4,835 nucleotide alterations). The greatest variation among the three taxa of *Trichuris* was in the COX1 region (42.4–58.9%), whereas least differences (24.3%–31.5%) were detected in the rrnL and rrnS subunits, respectively (Table 3).

Amino acid sequences inferred from individual mt protein genes of monkey-*Trichuris* were compared with those of human- and pig-*Trichuris*. The difference across amino acid sequences of the 13 protein genes between the monkey- and human-*Trichuris* was 28.52% (a total of 1015 amino acid alterations) and 38.28% (a total of 1364 amino acid alterations) between the monkey- and pig-*Trichuris*, respectively. The amino acid sequence differences among three taxa of *Trichuris* ranged from 13.6–62.5%, with COX1 being the most conserved and ATP8 the least conserved protein. Phylogenetic analyses of concatenated amino acid sequence data sets, using *T. spiralis* as an outgroup, revealed that the monkey-*Trichuris* was more closely related to the human-*Trichuris* than to representative *Trichuris* species from porcine and ruminant hosts, with absolute support (pp = 1.00) (Figure 2).

Comparison of the mt genomes of monkey-*Trichuris*, human-*Trichuris* and pig-*Trichuris* showed that the rrnL and rrnS were the two most conserved genes (Table 3). Sequence variation in part of the rrnL gene was assessed among four individuals of *Trichuris* from monkeys. The rrnL sequences of the four monkey-*Trichuris* individuals (GenBank accession nos. KC481232-KC481235) were of the same length (616 bp). Nucleotide variation among the four monkey-*Trichuris* individuals was detected at 15 sites (15/616; 2.44%). The four monkey-*Trichuris* sequences were aligned with 10 and six rrnL sequences (GenBank accession nos. AM993017-AM993032; [22]) reported previously for human- and pig-derived *Trichuris*, respectively. The alignment of the partial rrnL sequences revealed that all individuals of monkey-*Trichuris* differed at 140 nucleotide positions (140/430; 32.6%) when compared with human- and pig-*Trichuris*. Phylogenetic analysis of the rrnL

Figure 1. Structure of the mitochondrial genome for *Trichuris from the Francois’ Langur* (*Trichuris* sp.). Genes are designated according to standard nomenclature, except for the 22 tRNA genes, which are designated using one-letter amino acid codes, with numerals differentiating each of the two leucine- and serine-specifying tRNAs (L1 and L2 for codon families CUN and UUR, respectively; S1 and S2 for codon families AGN and UCN, respectively). “NCR-S” refers to a large non-coding region; “NCR-L” refers to a small non-coding region.  doi:10.1371/journal.pone.0066249.g001
sequence data from individual worms revealed strong support for the separation of monkey-\textit{Trichuris} from human-\textit{Trichuris} and pig-\textit{Trichuris} (Figure 3). Sequence variation was examined for both ITS-1 and ITS-2 of monkey-\textit{Trichuris}. The ITS-1 and ITS-2 sequences from four individual adults of monkey-\textit{Trichuris} were compared with those of human- and pig-derived \textit{Trichuris} [25]. Sequence variations were 0–0.7\% (ITS-1) and 0–0.9\% (ITS-2) among the three specimens of monkey-\textit{Trichuris}. However, the sequence differences were 21.4–22.3\% (ITS-1) and 22.4–23.7\% (ITS-2) between the monkey- and human-\textit{Trichuris}, and 56.5–57.0\% (ITS-1) and 43.6–45.5\% (ITS-2) between the monkey- and pig-\textit{Trichuris}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Gene or region} & \textbf{Positions and nt sequence lengths (bp)} & \multicolumn{3}{c|}{\textbf{Strand}} & \textbf{Initiation/termination codons} & \textbf{Anticodons} \\
\hline
\textbf{cox}1 & 1–1536 & 1–1545 & 1–1542 & H & ATG/TAG & ATG/TAA & ATG/TAG \\
\textbf{cox}2 & 1556–2230 & 1560–2234 & 1578–2258 & H & ATG/TAA & ATG/TAA & ATG/TAA \\
\textbf{rRNA–Leu(UUR) L\textsubscript{1}} & 2264–2328 (65) & 2251–2313 (63) & 2271–2332 (62) & H & TAA & \\
\textbf{rRNA–Glu (E)} & 2339–2396 (58) & 2318–2374 (57) & 2337–2393 (57) & H & TTC & \\
\textbf{nad}1 & 2413–3132 & 2397–3296 & 2415–3314 & H & ATT/TAA & ATAA & ATT/TAG \\
\textbf{Non–coding region (NCR–L)} & 3313–3436 (124) & 3297–3458 (162) & 3315–3438 (144) & H & \\
\textbf{rRNA–Lys (K)} & 3437–3501 (65) & 3459–3524 (66) & 3459–3521 (63) & H & TTT & \\
\textbf{nad}2 & 3508–4407 & 4406–3522 & 4414–3533 & L & ATG/TAA & ATAA & ATAA & ATAG \\
\textbf{rRNA–Met (M)} & 4468–4408 (61) & 4479–4419 (61) & 4485–4424 (62) & L & CAT & \\
\textbf{rRNA–Phe (F)} & 4463–4521 (59) & 4530–4474 (57) & 4546–4488 (59) & L & GAA & \\
\textbf{nad}5 & 6070–4523 & 6078–4531 & 6094–4538 & L & ATAA & ATAA & ATAA & ATAG \\
\textbf{Non–coding region (NCR–S)} & 7905–7962 (58) & 7905–7962 (58) & 7905–7962 (58) & H & TGT & \\
\textbf{cyt}b & 8273–9379 & 8278–9384 & 8278–9384 (50) & H & GCT & \\
\textbf{rrn}S & 9378–9429 (52) & 9383–9432 (50) & 9612–9666 (55) & H & \\
\textbf{rrn}L & 9422–10126 & 9425–10122 & 9425–10122 (57) & H & \\
\textbf{atp}6 & 10129–10185 (57) & 10124–10180 (57) & 10124–10180 (57) & H & TAC & \\
\textbf{cox}3 & 12001–12744 & 11975–12748 & 11975–12748 & H & ATG/TAA & ATAA & ATAA & ATAG \\
\textbf{rrNA–Trp (W)} & 12844–12779 (66) & 12817–12755 (63) & 12817–12755 (63) & H & TCA & \\
\textbf{rrNA–Gln (Q)} & 12852–12908 (57) & 12821–12874 (54) & 12821–12874 (54) & H & TTG & \\
\textbf{rrNA–Ile (I)} & 12992–12927 (66) & 12937–12871 (66) & 12937–12871 (66) & H & GAT & \\
\textbf{rrNA–Gly (G)} & 13057–12996 (62) & 13003–12947 (57) & 13003–12947 (57) & H & TCC & \\
\textbf{rrNA–Asp (D)} & 13063–13121 (59) & 13009–13067 (58) & 13009–13067 (58) & H & GTC & \\
\textbf{atp}8 & 13109–13273 & 13055–13219 & 13055–13219 & H & ATAG & ATAG & TTG/TAAG \\
\textbf{nad}3 & 13286–13624 & 13229–13570 & 13229–13570 & H & ATAG & ATAA & TAA \\
\textbf{Non–coding region (NCR–S)} & 13571–13663 (93) & 13887–14003 (117) & 13887–14003 (117) & H & TGA & \\
\textbf{rrNA–Ser UCN (S2)} & 13730–13782 (53) & 13664–13715 (52) & 13664–13715 (52) & H & GGT & \\
\textbf{rrNA–Asn (N)} & 13782–13836 (55) & 13715–13768 (54) & 14004–14055 (52) & H & TAG & \\
\textbf{rrNA–Leu UCN (L1)} & 13841–13907 (67) & 13776–13842 (67) & 13776–13842 (67) & H & TGC & \\
\textbf{rrNA–Ala (A)} & 13924–13983 (60) & 13845–13899 (57) & 13845–13899 (57) & H & \\
\textbf{rrNA–Cys (C)} & 14065–14003 (63) & 13979–13925 (55) & 13979–13925 (55) & H & GCA & \\
\textbf{rrNA–Tyr (Y)} & 14127–14068 (60) & 14046–13986 (50) & 14046–13986 (50) & H & TGT & \\
\hline
\end{tabular}
\end{table}
Table 3. Nucleotide and/or predicted amino acid (aa) sequence differences for mt protein-coding and ribosomal RNA genes among monkey-Trichuris (MT), human-Trichuris (Trichuris trichiura = TT) and pig-Trichuris (T. suis = TS).

| Gene/region | Nucleotide length (bp) | Nucleotide difference (%) | Number of aa | aa difference (%) |
|-------------|------------------------|---------------------------|--------------|------------------|
|             | MT | TT | TS | MT/TT | MT/TS | TT/TS | MT | TT | TS | MT/TT | MT/TS | TT/TS |
| atp6        | 822 | 828 | 828 | 36.8 | 40.2 | 39.6 | 273 | 275 | 275 | 40.0 | 49.5 | 49.5 |
| nad1        | 900 | 900 | 900 | 29.6 | 32.8 | 33.1 | 299 | 299 | 299 | 28.1 | 32.4 | 33.1 |
| nad2        | 900 | 885 | 882 | 34.7 | 39.2 | 34.1 | 299 | 294 | 293 | 31.8 | 44.2 | 40.8 |
| nad3        | 339 | 342 | 342 | 32.7 | 38.6 | 33.6 | 112 | 113 | 113 | 27.4 | 35.4 | 31.9 |
| nad4        | 1212 | 1212 | 1209 | 31.3 | 41.2 | 40.9 | 403 | 403 | 402 | 31.3 | 55.1 | 58.3 |
| nad4L       | 261 | 258 | 252 | 28.7 | 35.6 | 38.0 | 86  | 85  | 83  | 36.0 | 46.5 | 42.4 |
| nad5        | 1548 | 1548 | 1557 | 34.6 | 38.6 | 35.9 | 515 | 515 | 518 | 37.5 | 47.1 | 42.5 |
| nad6        | 468 | 477 | 471 | 32.3 | 35.7 | 33.1 | 155 | 158 | 156 | 35.4 | 45.6 | 38.6 |
| cox1        | 1536 | 1545 | 1542 | 24.2 | 25.0 | 25.4 | 511 | 514 | 513 | 13.6 | 16.6 | 13.6 |
| cox2        | 675 | 675 | 671 | 24.3 | 31.6 | 30.7 | 224 | 224 | 226 | 18.7 | 31.0 | 28.8 |
| cox3        | 774 | 774 | 777 | 29.2 | 31.0 | 35.5 | 257 | 257 | 258 | 25.7 | 30.6 | 34.5 |
| cyt b       | 1107 | 1107 | 1113 | 26.1 | 29.0 | 27.7 | 368 | 368 | 370 | 20.9 | 27.3 | 26.2 |
| atp8        | 165 | 165 | 171 | 42.4 | 50.9 | 47.4 | 54  | 54  | 56  | 55.6 | 58.9 | 62.5 |
| rrnS        | 705 | 698 | 712 | 25.4 | 24.3 | 24.6 | –   | –   | –   | –   | –   | –   |
| rrnL        | 1007 | 1011 | 1011 | 25.1 | 31.5 | 25.1 | –   | –   | –   | –   | –   | –   |

Discussion

To date, more than 20 *Trichuris* species have been described from various mammalian hosts based on the microscopic features of the adult worms [42]. Some studies (e.g., [43,44]) have claimed that male spicule and body lengths are useful morphological parameters for the differentiation of *Trichuris* species. However, other studies have shown that these measurements are not necessarily reliable for specific identification [15]. For instance, Cutillas et al. (2009) [15] observed that the spicule lengths of *T. trichiura* and *T. suis* overlapped. While other workers considered that the presence of pericoacal papillae might be useful for species determination [45], also this criterion does not appear to allow accurate identification/delineation [15]. Clearly, these studies show that morphological characters or morphometrics should be interpreted with caution. For this reason, we employed here a molecular genetic approach, logically extending previous studies [22–26], so that comparative genetic analyses could be conducted.

The present investigation shows clear genetic distinctiveness between *Trichuris* from the François’ langur and *Trichuris* from humans and livestock animals (i.e., *T. suis*, *T. ovis* and *T. discolor*) (Figure 2). Our and previous findings [22–27] support the contention that each *Trichuris* species has a very specific affiliation with a particular host species [16], although, to date, only small numbers of adult worms have been studied molecularly. Clearly, larger population genetic and molecular epidemiological studies should be conducted using the mt and nuclear markers defined in this and previous studies [22–28] to further test this hypothesis.

The sequence difference in the inferred mt proteome between monkey- and human-*Trichuris* was 29.4%, and sequence variation among individual worms from each host species was low (0–2.4%), suggesting that these parasites are separate species. This proposal was further supported by phylogenetic analysis (cf. Figure 3). Previous studies [7–13] have indicated that many non-human primates, such as *Colobus guereza*, *Macaca fascicularis*, *M. silenus*, *Papio anubis*, *P. hamadryas ursinus* and *Theropithecus gelada*, can harbour *Trichuris*. However, the specific identity, host specificity and zoonotic potential of each operational taxonomic unit [13] of *Trichuris* from each of these host species are unknown. Based on molecular findings to date for *Trichuris* from other animal species [22–28,46–51], we anticipate that each primate species harbours its own species of *Trichuris*, but, clearly, this proposal requires rigorous testing.

**Trachypithecus franc¸oisi** is a threatened/endangered species of primate nearing extinction [52]. Populations of this langur have
been on the decline for the past 30 years. For instance, the populations in Guangxi province, China, crashed from 4000–5000 individuals in 1980 to a mere 307 in 2002–2003 [53]. The main factors linked to this decline have been hunting, habitat destruction and harvesting of langur organs for the preparation of traditional medicines [54,55]. Another likely threat to the destruction and harvesting of langur organs for the preparation of traditional medicines [54,55]. Another likely threat to the conservation of traditional medicines [54,55].

The analyses of mitochondrial rnr, sequence data were carried out by Bayesian inference (BI), using Trichinella spiralis as the outgroup. Posterior probabilities (pp) values of <0.9 are not shown.

**Figure 3. Inferred genetic relationships of four individual specimens of from the François’ langur (**Trichuris sp.) with those of **Trichuris suis** (n = 6) and **T. suis** (n = 10) from China. The analyses of mitochondrial rnr sequence data were carried out by Bayesian inference (BI), using Trichinella spiralis as the outgroup. Posterior probabilities (pp) values of <0.9 are not shown.

doi:10.1371/journal.pone.0066249.g003

**References**

1. Hotez PJ, Fenwick A, Savioli L, Molyneux DH (2009) Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373: 1570–1575.
2. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, et al. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367: 1215–1224.
3. Roepstorff A, Mejer H, Nejsum P, Thamsborg SM (2011) Helminth parasites in pigs: new challenges in pig production and current research highlights. Vet Parasitol 179: 236–246.
4. Traversa D (2011) Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like Trichuris suis? Parasit Vectors 4: 32.
5. Khalafalla RE, Elseify MA, Elbahy NM (2011) Seasonal prevalence of gastrointestinal nematode parasites of sheep in Northern region of Nile Delta, Egypt. Parasitol Res 108: 337–340.
6. Cliffe LJ, Grencis RK (2004) The Trichuris suis system: a paradigm of resistance and susceptibility to intestinal nematode infection. Adv Parasitol 57: 255–307.
7. Leecke B, Dornay P, Geurden T, Vercammen F, Verbruggen J (2007) Gastrointestinal protozoa in non-human primates of four zoological gardens in Belgium. Vet Parasitol 148: 236–246.
8. Lin K, Li Z, Li L, Zhou J, Zhang R, et al. (2011) Investigation on the intestinal parasites infection in conventional (CV) macaques. Fujian Anim Husbandry Vet Med 6: 1–4 (in Chinese).
9. Legesse M, Erko B (2004) Zoonotic intestinal parasites in *Papio anubus* (baboon) and *Cercopithecus aethiops* (vervet) from four localities in Ethiopia. Acta Trop. 90: 337–340.
10. *Parasito* (2011) Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like Trichuris suis? Parasit Vectors 4: 32.
11. Khalafalla RE, Elseify MA, Elbahy NM (2011) Seasonal prevalence of gastrointestinal nematode parasites of sheep in Northern region of Nile Delta, Egypt. Parasitol Res 108: 337–340.
12. Cliffe LJ, Grencis RK (2004) The Trichuris suis system: a paradigm of resistance and susceptibility to intestinal nematode infection. Adv Parasitol 57: 255–307.
13. Levecke B, Dornay P, Geurden T, Vercammen F, Verbruggen J (2007) Gastrointestinal protozoa in non-human primates of four zoological gardens in Belgium. Vet Parasitol 148: 236–246.
14. Lin K, Li Z, Li L, Zhou J, Zhang R, et al. (2011) Investigation on the intestinal parasites infection in conventional (CV) macaques. Fujian Anim Husbandry Vet Med 6: 1–4 (in Chinese).
15. *Parasito* (2011) Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like Trichuris suis? Parasit Vectors 4: 32.
16. Khalafalla RE, Elseify MA, Elbahy NM (2011) Seasonal prevalence of gastrointestinal nematode parasites of sheep in Northern region of Nile Delta, Egypt. Parasitol Res 108: 337–340.
17. Cliffe LJ, Grencis RK (2004) The Trichuris suis system: a paradigm of resistance and susceptibility to intestinal nematode infection. Adv Parasitol 57: 255–307.
comparison with parasitological data from man in the region, J Med Primatol 32: 341–345.

13. Ravasi DF, O’Rrain MJ, Adams VJ, Appleton CC (2012) A coprological survey of the protozoan and nematode parasites of free-ranging chacma baboons (Papio ursinus) in the southwestern Cape, South Africa. J Wildl Res 42: 35–44.

14. Ooi HK, Tenora F, Itoh K, Kaniya M (1993) Comparative study of Trichurus trichuria from non-human primates and from man, and their difference with T. suis. J Vet Med Sci. 55: 363–366.

15. Cutillas C, Callejon R, Rojas MD, Torres B, Ubeda JM (2009) Trichuris suis and Trichuris trichiura are different nematode species. Acta Trop 109: 299–307.

16. Beer RJ (1976) The relationship between Trichurus trichiura (Linnaeus 1758) of man and Trichurus suis (Schröpf 1788) of the pig. Res Vet Sci 28: 47–54.

17. Bain BM (2002). Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int J Parasitol 32: 527–531.

18. Gasser RB (2006) Molecular tools—advances, opportunities and prospects. Vet Res 37: 136–146.

19. Gasser RB, Butt NJ, Chilton NB, Hunt P, Beveridge I (2008) Toward practical, DNA-based diagnostic methods for parasitic nematodes of livestock-biomass and biotechnological implications. Biotechnol Adv 26: 325–334.

20. Jex AR, Warschubenach A, Hu M, van Wyk JA, Beveridge I, et al. (2009) The mitochondrial genomes of Ancylostoma caninum and Bunostomum phlebotomum two hookworms of animal health and zoonotic importance. BMC Genomics 10: 79.

21. Jex AR, Littlewood DT, Gasser RB (2010) Toward next-generation sequencing of mitochondrial genomes—focus on parasitic worms and animals and biotechnological implications. Biotechnol Adv 28: 151–159.

22. Liu GH, Gasser RB, Su A, Nejsum P, Peng L, et al. (2012) Clear genetic distinctiveness between human- and pig-derived Trichuris based on analyses of mitochondrial datasets. PLoS Negl Trop Dis 6: e1339.

23. Liu GH, Wang Y, Xu MJ, Zhou DH, Ye YG, et al. (2012) Characterization of the complete mitochondrial genomes of two whipworms Trichuris suis and Trichuris duodenale (Nematoda: Trichuridae). Infect Genet Evol 12: 1635–1641.

24. Nissen S, Al-Jubury A, Hansen TV, Olsen A, Christensen H, et al. (2012) Genetic analysis of Trichuris suis and Trichuris trichiura recovered from humans and pigs in a sympatric setting in Uganda. Vet Parasitol 188: 68–77.

25. Liu GH, Zhou W, Nisbet AJ, Xu MJ, Zhou DH, et al. (2012) Characterization of Trichurus trichiura from humans and T. suis from pigs in China using internal transcribed spacers of nuclear ribosomal DNA. J Helminthol. In press.

26. Cutillas C, Oliveros R, de Rojas M, Guevara DC (2002) Determination of Trichuris muris from murid hosts and T. arvicolae (Nematoda: Arvicolidae) from arvicolid rodents by amplification and sequencing of the ITS-1–5.8S-ITS2 segment of the nuclear ribosomal DNA. Parasitol Res 89: 574–582.

27. Cutillas C, de Rojas M, Ariza C, Ubeda JM, Guevara D (2007) Molecular identification of Trichuris vulpis and Trichuris suis isolated from different hosts. Parasitol Res 100: 583–589.

28. Ravasi DF, O’Rrain MJ, Davis F, Illing N (2012) Phylogenetic evidence that two distinct Trichuris genotypes infect both humans and non-human primates. PLoS One. 7: e44187.

29. Hu G, Wei Y (2002) Population decline and habitats destruction of Francois’ Langur Trachypithecus francoisi in Guangxi Nature Reserve. Southwestern Guangxi, China. In: Abstr 19th Cong Int Primat Soc. Beijing, China, 74–75.

30. Gasser RB, Hu M, Chilton NB, Campbell BE, Jex AR, et al. (2006) Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. Nat Protoc 1: 3121–3129.

31. Gasser RB, Zhu XQ, McMullan DP (1999) NADH dehydrogenase subunit 1 and cytochrome c oxidase subunit 1 sequences compared for members of the genus Fasciola (Cestoda). Int J Parasitol 29: 1905–1910.

32. Hu M, Jex AR, Campbell BE, Gasser RB (2007) Long PCR amplification of the entire mitochondrial genome from individual helminths for direct sequencing. Nature Protoc 2: 2339–2344.

33. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882.

34. Lowe TM, Eddy SR (1997) RNASEAN—SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res 25: 955–964.

35. Hu M, Chilton NB, Gasser RB (2002) The mitochondrial genomes of the human hookworms, Ancylostoma duodenale and Necator americanus (Nematoda: Saccodontidae). Int J Parasitol 32: 145–158.

36. Zhu X, Chilton NB, Jacobs DE, Boes J, Gasser RB (1999) Characterisation of Ascaris from human and pig hosts by nuclear ribosomal DNA sequences. Int J Parasitol 29: 469–476.
Author/s:
Liu, G.-H; Gasser, R.B; Nejsum, P; Wang, Y; Chen, Q; Song, H.-Q; Zhu, X-Q

Title:
Mitochondrial and Nuclear Ribosomal DNA Evidence Supports the Existence of a New Trichuris Species in the Endangered Francois' Leaf-Monkey

Date:
2013-06-20

Citation:
Liu, G.-H., Gasser, R. B., Nejsum, P., Wang, Y., Chen, Q., Song, H.-Q. & Zhu, X.-Q. (2013). Mitochondrial and Nuclear Ribosomal DNA Evidence Supports the Existence of a New Trichuris Species in the Endangered Francois' Leaf-Monkey. PLOS ONE, 8 (6), https://doi.org/10.1371/journal.pone.0066249.

Persistent Link:
http://hdl.handle.net/11343/264984

License:
CC BY