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Research paper

Insights into the molecular systematics of *Trichuris* infecting captive primates based on mitochondrial DNA analysis

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ABSTRACT

Nematodes belonging to the *Trichuris* genus are prevalent soil-transmitted helminths with a worldwide distribution in mammals, while humans are mainly affected in areas with insufficient sanitation such as in Africa, Asia and South America. Traditionally, whipworms infecting primates are referred to *Trichuris trichiura*, but recent molecular and morphological evidence suggests that more than one species may be able to infect humans and non-human primates. Here, we analyzed the genetic diversity and phylogeny of *Trichuris* infecting five different non-human primate species kept in captivity using sequencing of three mitochondrial genes (*cox*1, *rrn*L and *cob*).

Phylogenetic analyses of both single and concatenated datasets suggested the presence of two main evolutionary lineages and several highly supported clades likely existing as separate taxa. The first lineage included *Trichuris* infecting the mantled guereza (*Colobus guereza kikuyensis*), the chacma baboon (*Papio ursinus*) and the green monkeys (*Chlorocebus* spp.), clustering together with *Trichuris suis*; the second lineage included *Trichuris* infecting the Japanese macaque (*Macaca fuscata*) and the hamadryas baboon (*Papio hamadryas*), clustering together with *Trichuris* spp. infecting humans. These results were supported by the genetic distance between samples, which suggested that at least two taxa are able to infect macaques, baboons and humans.

The present study improves our understanding of the taxonomy and evolutionary relationships among *Trichuris* spp. infecting primates. It moreover suggests that multiple *Trichuris* spp. may circulate among host species and that *Trichuris* in non human primates (NHPs) may be zoonotic. Further studies are important to better understand the epidemiology of *Trichuris* in primates and for implementing appropriate control and/or conservation measures.

1. Introduction

Nematodes of the genus *Trichuris*, commonly known as whipworms, are intestinal parasites infecting a large number of mammalian species (Anderson, 2000). Transmission is direct, and hosts become infected by ingestion of food, water and/or soil contaminated with embryonated eggs. The eggs hatch in the intestine, where L1 larvae are released and penetrate the epithelial cells of the large intestine. Here, they develop into the adult stage, and after mating, unembryonated eggs are released from the females and further into the environment with host faeces.

Clinically, trichurosis is associated with dysentery, bloody diarrhoea, rectal prolapse, and (for humans) nausea and cognitive impairment but clinical symptoms depend on infection level (Hotez et al., 2007).

Around one hundred *Trichuris* species have been recognized (Yamaguti, 1961), and these have traditionally been described based on morphology and the host species from which they were isolated. *Trichuris trichiura* is one of the four highly prevalent soil-transmitted helminths of human clinical importance, infecting about five hundred million people in mainly South and South-East Asia, Sub-Saharan Africa and Latin America in areas with poor sanitation (World Health Organization, 2013; Pullan et al., 2014).

It has been suggested that humans also occasionally can be infected by *T. vulpis* (Froelich, 1789), and *T. suis* (Schrank, 1788), and therefore,

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these are considered zoonotic species (Areekul et al., 2010; Nissen et al., 2012; Ravasi et al., 2012). Moreover, the recent use of whipworm administration as a therapeutic option for autoimmune diseases like inflammatory bowel disease (Schölmerich, 2013; Huang et al., 2018), has stimulated investigations into the taxonomic status of *Trichuris* from human and non-human primates (NHPs) and other related species and their zoonotic potential.

Traditionally, whipworms in primates have been referred to as *T. trichiura* (or more conservatively referred to *Trichuris* spp.), but recent molecular evidence suggests a more complex scenario, with several *Trichuris* taxonomic entities related to primates with different degrees of host preference. Ravasi et al. (2012) suggested potential cross-infection of *Trichuris* sp. between baboons and humans in South Africa, and phylogenetic analysis advocated for the existence of two distinct lineages infective to both hosts. Other studies have also suggested that multiple taxonomic entities of *Trichuris* circulate among NHPs with some taxa clustering in evolutionary clades with whipworms from humans, while others are more closely related to *T. suis* (Liu et al., 2013; Cutillas et al., 2014; Callejón et al., 2017). It therefore remains unknown how many species infect NHPs and how they are related to *Trichuris* in humans. However, current data suggest that some species may be zoonotic (Nissen et al., 2012; Ghai et al., 2014; Cavallero et al., 2015; Doležalová et al., 2015; Hawash et al., 2015, 2016). To this end, a molecular study of both beta-tubulin genes and ITS-2 regions of *Trichuris* from both humans and baboons (*Papio anubis, Papio hamadryas*) revealed no genetic differences between host species (Hansen et al., 2013). Also, a feral population of African green monkeys (*Chlorocebus sabaeus*) living in Saint Kitts was recently suggested as reservoir host of *T. trichiura* in humans, spurring a one-health approach to curtailing enteric parasitic infections in human populations in the area (Hawash et al., 2016; Yao et al., 2018). Lastly, Xie et al. (2018) emphasized a puzzling taxonomy of *T. trichiura* based on ITS and *cox*1 sequences, recognizing seven genetically distinct subgroups of whipworms from primates of China.

Beside the uncertainty regarding the taxonomic status of *Trichuris* from NHPs, these data also suggest a zoonotic potential of *Trichuris*. NHPs represent a reservoir for several known zoonotic infectious diseases (LeBreton et al., 2014; Karim et al., 2015; Narat et al., 2017), and the identification of origin and potential transmission of such infective agents from both wild and captive primates is of major importance for public health and occupational risk.

Here, we aimed to explore the evolutionary relationships among *Trichuris* infecting primates using three mitochondrial markers (*cox*1, *rrn*L and *cob*). The level of genetic diversity and phylogenetic relationships were estimated for *Trichuris* collected from five different captive NHP species.

2. Materials & methods

2.1. Isolation of material

Adult *Trichuris* were collected from intestinal caeca during necropsy of the following primate species living in European zoological parks: the Japanese macaque (*Macaca fuscata*), the grivet (*Chlorocebus aethiops*), the African green monkey (*C. sabaeus*), the mantled guereza (*Colobus guereza*), and the hamadryas baboon (*P. hamadryas*). Nematodes were washed in saline (0.9%), morphologically identified as *Trichuris* sp. according to Skrjabin et al. (1957) and Ooi et al. (1993), and fixed in 70% ethanol at room temperature until DNA extraction.

Total genomic DNA was isolated from 45 individual male and female adult specimens (Table 1), using the Wizard Genomic DNA Purification kit (Promega), according to the manufacturer's instructions. Fragments of the three mitochondrial genes, ribosomal RNA (*rrn*L), cytochrome *b* (*cob*) and subunit 1 of cytochrome oxidase (*cox*1), were amplified by PCR as described by Liu et al. (2012) and Callejón et al. (2013) using the following primers: rrnLF (5′-TAAATGGCCGTCGTAA CGTGACTGT-3′) and rrnLR (5′-AAAGAGAATCCATTCTATCTCGCA ACG-3′) were used for *rrn*L; D769 (5′-GAGTAATTTTTATAATRCGRGA-AGT-3′) and D770 (5′-AATTTTCAGGRTCTCTRCTTCAAT -3′) were used for *cob*; CORA (5′-ACYACATAGTAGGTRTCATG-3′), and HC02198 F (5′-TGATTTTTTGGTCACCCTGAAGTTTA-3′) were used for *cox*1 amplification.

Negative (no template DNA) controls were included in all runs. Successful amplification was confirmed using 1.5% agarose gel electrophoresis and ethidium bromide staining. PCR products were purified using Sure Clean (Bioline) and shipped to an external service for sequencing (MWG Eurofins Operon).

2.2. Sequencing, evolutionary distance and phylogenetic analyses

Electropherograms were manually checked using Tracer, and sequences were aligned using ClustalW, both of which are implemented in MEGA7 (Kumar et al., 2016); for the rrnL dataset, an alignment was developed using WebPrank (Löytynoja and Goldman, 2010) to improve functional constraints of secondary structure. For comparison, additional mitochondrial sequences from *Trichuris* infecting primates and pigs from different geographical regions were included in the multiple sequence alignment and two species of *Trichinella* were included as outgroups (Table 1). A dataset for each mtDNA gene named DatasetCO1, DatasetrrnL and DatasetCOB were generated, together with a concatenated dataset with partitionable sequences belonging to the same specimens obtained in the present study and retrieved from GenBank.

Evolutionary distances were estimated using MEGA7 by the mean of genetic *p-distance* for all datasets, calculated as the total percentage of sequence differences for each pair, as this measure may be useful for identification of cryptic species (*sensu* Blouin, 2002).

Both for single genes and the concatenated dataset spanning *cox1-rrnL-cob* regions, model test analyses were run partitioned by each gene, and ModelTest (implemented in MEGA7) was used to compare the fit of nucleotide substitution models, according to the lowest BIC (Bayesian information criterion) score (Nei and Kumar, 2000; Kumar et al., 2016). Bayesian Inferences (BI) was performed on both single and concatenated datasets (four datasets in total) using the following models: TN93 + G + I for DatasetCO1; HKY + I for DatasetrrnL, HKY + G + I for DatasetCOB. The BEAST software (Drummond et al., 2012) was used under the "birth-death with incomplete sampling" scenario (Kühnert et al., 2016); datasets were run twice for 10^6 generations, with a sampling chain for every 1000 generations. The Yule prior, suitable for datasets comprising different species, was used as a tree prior with a random starting tree. Posterior probability values (BPP) shown in the Bayesian consensus trees were determined after discarding trees from the burn-in period, estimated to include the first 100 generations using TreeAnnotator (Drummond et al., 2012). Tracer v.1.6 (Rambaut et al., 2018) was used to analyze log files and to check the reliability of the MCMC chains. Consensus trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Sequences obtained in the present study were submitted to GenBank, and accession numbers are available in Table 1.

3. Results

3.1. Sequencing

Trichuris-specific DNA was amplified from five different primate hosts, with 42 specimens amplified with fragments of about 450bp, 41 specimens with fragments of 500bp and 30 specimens with fragments of 550bp for *cox*1, *rrn*L and *cob* mitochondrial regions, respectively, visualized by gel electrophoresis. After trimming sequence ends in accordance with the length of available sequence in GenBank, DatasetCO1 included 72 sequences for a total alignment length of 341 basepairs (bp); DatasetrrnL included 145 sequences and an alignment length of

Table 1
Material analyzed in the present study for phylogenetic inferences based on the three mitochondrial markers *cox*1, *rrn*L and *cob*. Information on parasite species as defined by authors, host species, GenBank accession numbers, Specimen codes and references are available.

Parasite species	Host species	GenBank accession number	Specimen code	Authors
DatasetCO1				
Trichuris trichiura	*Homo sapiens*	GU385218	H1	Liu et al. (2012)
T. trichiura	*Homo sapiens*	AP017704	H2	Kikuchi et al. (unpublished)
T. trichiura	*Homo sapiens*	KT449826	H3	Hawash et al. (2015)
Trichuris sp.	*Homo sapiens*	JF690962	H4	Doležalová et al. (2015)
T. trichiura	*Colobus sp.*	FR846241	C6	Callejón et al. (unpublished)
T. colobae	*Colobus guereza*	HE653116-HE653120	C1-5	Callejón et al. (2013)
T. colobae	*Colobus guereza*	MK762948-49	Cg1-2	Present study
Trichuris sp.	*Chlorocebus aethiops*	MK762929-42	Ca3,5,6,8-15	Present study
Trichuris sp.	*Chlorocebus sabaeus*	MK762923-28	Cs1-6	Present study
Trichuris sp.	*Macaca fuscata*	MK762905-7	Mfa1,5,6	Present study
		MK762908-22	Mfb2-4,7, 9-19	
T. ursinus	*Papio ursinus*	LT627353	PU1	Callejón et al. (2017)
Trichuris sp.	*Papio hamadryas*	JF690963	Ph1	Doležalová et al. (2015)
Trichuris sp.	*Papio hamadryas*	MK762943-47	Ph91-95	Present study
T. trichiura	*Papio sp*	HG003692	TtriP1	Callejón and Cutillas (unpublished)
T. suis	*Sus scrofa scrofa*	HQ183740-42	S1-3	Liang et al. (unpublished)
		HQ204208-10	S4-6	
		HE653124-29	S7-12	Zhang et al. (unpublished)
				Callejón et al. (2013)
DatasetrrnL				
T. trichiura	*Homo sapiens*	GU385218	H1	Liu et al. (2012)
		AM993017-22	H2-7	
T. trichiura	*Homo sapiens*	KP781898-KP781912	H8-21	Meekums et al. (2015)
T. trichiura	*Homo sapiens*	KU524541-KU524557	H22-38	Hawash et al. (2016)
Trichuris sp.	*Chlorocebus aethiops*	MN088565-77	Ca1-3,5,6,8-14	Present study
Trichuris sp.	*Chlorocebus sabaeus*	MN088559-64	Cs1-6	Present study
Trichuris sp.	*Chlorocebus sabaeus*	KU524595- KU524606	C1-12	Hawash et al. (2016)
T. colobae	*Colobus guereza*	MN088583-85	Cg1-3	Present study
Trichuris sp.	*Macaca fuscata*	MN088542-43	Mfa3,5	Present study
		MN088544-58	Mfb2-4,6-8, 11-14,16-19	
Trichuris sp.	*Papio sp.*	KU524558- KU524594	P1-37	Hawash et al. (2016)
Trichuris sp.	*Papio hamadryas*	MN088578-82	Ph92-95	Present study
Trichuris sp.	*Trachypithecus francoisi*	KC481232-35	TF 1-4	Liu et al. (2013)
T. suis	*Sus scrofa*	KP781894-97	S1-4	Hawash et al. (2016)
		KU524537-40	S5-8	
DatasetCOB				
T. trichiura	*Homo sapiens*	GU385218	H1	Liu et al. (2012)
T. trichiura	*Homo sapiens*	KT449826	H2	Hawash et al. (2015)
Trichuris sp.	*Chlorocebus aethiops*	MK914564-72	Ca3,5-6 8-11,14,15	Present study
Trichuris sp.	*Chlorocebus sabaeus*	MK914562-63	Cs3,4	Present study
T. colobae	*Colobus guereza*	LM994704	Cg1	Callejón et al. (2015)
T. colobae	*Colobus guereza*	MK914578-79	Cg2-3	Present study
Trichuris sp.	*Macaca fuscata*	MK914550-53	Mfa3-6	Present study
		MK914554-61	Mfb2-4,7,10-13	
T. ursinus	*Papio ursinus*	LT627357-60	PU1-4	Callejón et al. (2017)
Trichuris sp.	*Papio hamadryas*	KT449824	P1	Hawash et al. (2015)
Trichuris sp.	*Papio hamadryas*	MK914573-77	Ph91-95	Present study
Trichuris sp.	*Papio anubis*	KT449825	P2	Hawash et al. (2015)
Trichuris sp.	*Papio sp.*	LM994703	P3	Callejón et al. (2015)
T. suis	*Sus scrofa scrofa*	KT449822-23	S1-2	Hawash et al. (2015)
Outgroup species				
Trichinella britovi		KM357413		Mohandas et al. (2014)
Trichinella spiralis		AF293969		Lavrov and Brown (2001)

403 bp, while DatasetCOB included 41 sequences and an alignment length of 477 bp.

3.2. Evolutionary distance

Mean genetic distances were estimated over sequence pairs within and between groups, developed based on host affiliation.

Within-host species analyses of *Trichuris* sequences revealed variable ranges of genetic distances (Table 2), with the lowest *p-distance* obtained for *Trichuris* from *C. aethiops* (0.0%–0.2%), and the highest distances reported for *T. trichiura* from humans (0.0%–14.6%) and baboons (0.5%–16.8%).

Table 2
Within-group estimates of evolutionary divergence based on *p-distance* for DNA sequences of *Trichuris* spp. analyzed in the present study.

	DatasetCO1	DatasetrrnL	DatasetCOB	Concatenated
Human	0.146	0.052	0	0.138
Pig	0.054	0.010	0.068	0.073
Baboon	0.066	0.005	0.168	0.057
Macaque	0.045	0.018	0.065	0.035
Colobus	0.003	0.059	0	0.020
Grivet	0	0	0.002	0.002
Green Monkey	0.017	0.091	0.022	0.013

Table 3
: Between-groups estimates of evolutionary divergence based on *p-distance* for DNA sequences of *Trichuris* spp. analyzed in the present study for a) DatasetCO1, b) DatasetrrnL, c) DatasetCOB and d) Concatenated.

	Human	Pig	Baboon	Macaque	Colobus	Grivet	**Green** Monkey
Human							
Pig	a) 0.207						
	b) 0.215						
	c) 0.268						
	d) 0.239						
Baboon	a) 0.149	a) 0.208					
	b) 0.055	b) 0.212					
	c) 0.191	c) 0.267					
	d) 0.081	d) 0.246					
Macaque	a) 0.152	a) 0.215	a) 0.122				
	b) 0.062	b) 0.208	b) 0.056				
	c) 0.138	c) 0.279	c) 0.161				
	d) 0.109	d) 0.241	d) 0.104				
Colobus	a) 0.214	a) 0.207	a) 0.229	a) 0.223			
	b) 0.222	b) 0.166	b) 0.206	b) 0.221			
	c) 0.281	c) 0.229	c) 0.266	c) 0.277			
	d) 0.242	d) 0.203	d) 0.245	d) 0.244			
Grivet	a) 0.232	a) 0.203	a) 0.210	a) 0.212	a) 0.188		
	b) 0.200	b) 0.130	b) 0.188	b) 0.203	b) 0.137		
	c) 0.258	c) 0.233	c) 0.251	c) 0.261	c) 0.241		
	d) 0.238	d) 0.198	d) 0.236	d) 0.229	d) 0.196		
Green Monkey	a) 0.233	a) 0.201	a) 0.212	a) 0.212	a) 0.187	a) 0.029	
	b) 0.103	b) 0.186	b) 0.066	b) 0.105	b) 0.184	b) 0.131	
	c) 0.261	c) 0.220	c) 0.256	c) 0.271	c) 0.250	c) 0.057	
	d) 0.241	d) 0.192	d) 0.241	d) 0.237	d) 0.200	d) 0.036	

Between-host species distances obtained from sequence analyses showed ranges of 2.9% to 13% for *Trichuris* infecting *Chlorocebus* spp. and from 13% up to 27% for all others NHPs pairs (Table 3). List of values obtained for all datasets is reported in Supplementary Material S1.

3.3. Phylogeny

All trees obtained by BI identified two main phylogenetic lineages (clades) and, except for stochastic events most likely due to uneven gene coverage among different taxa, several highly supported groups.

Giving the higher number of sequences included in the DatasetrrnL compared to the other datasets, we assumed this consensus tree as the most representative and will be discussed in detail (Fig. 1). Two main clades were obtained and named according to the nomenclature used in previous studies (Ravasi et al., 2012; Callejón et al., 2013; Cavallero et al., 2015): the first named Clade 1CP-GOB grouped *Trichuris* from *Chlorocebus sabaeus* and *C. aethiops, T. suis*, and *T. colobae*, with maximum support (1.00 pp); the second named Clade 2 included four branches. Within the latter, subclade 2A was represented by *Trichuris* here analyzed from *P. hamadryas* and *M. fuscata*, together with specimens from humans, *C. sabaeus* and *Papio* sp. (0.99 pp); a second branch named subclade MF including *Trichuris* from *M. fuscata* (1.00 pp); the third was represented by *T. trichiura* from humans (subclade H); lastly *Trichuris* infecting *Trachypithecus francoisi* was the sister branch.

The phylogeny inferred on DatasetCO1 (Fig. 2A) confirmed the existence of the two clades, with slight differences in internal branching pattern. The Clade 1 CP-GOB (0.98 pp) showed *T. suis* (1.00 pp) as the sister branch of specimens from primates (0.88 pp), including *Trichuris* from *Chlorocebus* (1.00 pp), *T. ursinus* and *T. colobae*.

Clade 2 (1.00 pp) comprised the subclade MF, the subclade 2A, both related to the third branch represented by *T. trichiura* from humans named subclade H (0.96 pp).

The translated alignment of DatasetCO1 resulted in 113 codons showing several fixed amino acid (AA) polymorphisms, reflecting the branching pattern observed in phylogenetic tree and corresponding to clades and subclades previously described (specific partial *cox*1 polymorphism patterns are described in Supplementary material S2a). All *Trichuris* AA sequences from NHPs, one sequence from a human host, and *T. suis* exhibited an 'I' in position 80 instead of the 'V' that is observed in *T. trichiura* of human origin.

BI of DatasetCOB displayed a tree with the two main lineages represented by Clade 1CP-GOB and Clade 2, supported by high pp values (Fig. 2B). The main differences from DatasetrrnL topology are: i) the existence of a branch within Clade 1 composed of *T. colobae* and *T. suis* (0.99 pp) and ii) a weakly supported node (0.76 pp) within Clade 2, clustering subclade MF (1.00 pp) and few *Trichuris* sequences from *M. fuscata* and from *Papio* spp. (subclade 2A), both related to *T. trichiura* from humans and baboons. The translated AA alignment of DatasetCOB resulted in 159 codons showing several fixed polymorphisms mostly according to the phylogenetic branching pattern (S2b).

Phylogenetic inference based on the concatenated dataset included 32 sequences in a 1231 bp alignment. The consensus tree supported again the existence of the two main evolutionary lineages previously recognized (Fig. 2C). The Clade 1CP-GOB was again obtained, and Clade 2 had three well defined branches, similarly to the branching pattern observed in DatasetCOB. In the present study, whipworm DNA sequences from human hosts were observed only in Clade 2.

4. Discussion

Recent molecular investigations challenged the classic definition of *T. trichiura*, suggesting the existence of more than one taxonomic entity infecting primate species, with no clear definition of species boundaries and of potential of transmission to humans.

The topology of Clade 1CP-GOB including *Trichuris* from pigs and primates has previously been described (Ravasi et al., 2012; Callejón et al., 2013; Cutillas et al., 2014; Cavallero et al., 2015; Callejón et al., 2017), suggesting genetic similarities between *T. ursinus*, *T. suis* and *T. colobae* (Cutillas et al., 2014; Callejón et al., 2017). In the present study, the existence of this clade is supported by very high pp values for all phylogenetic inferences (DatasetCO1, pp = 0.98; DatasetrrnL, DatasetCOB and Concatenated, pp = 1.00).

Hawash et al. (2016) used coalescent analyses to explore the evolutionary relationships among whipworms from primates and pigs and suggested an African origin of human *T. trichiura*, which may have been

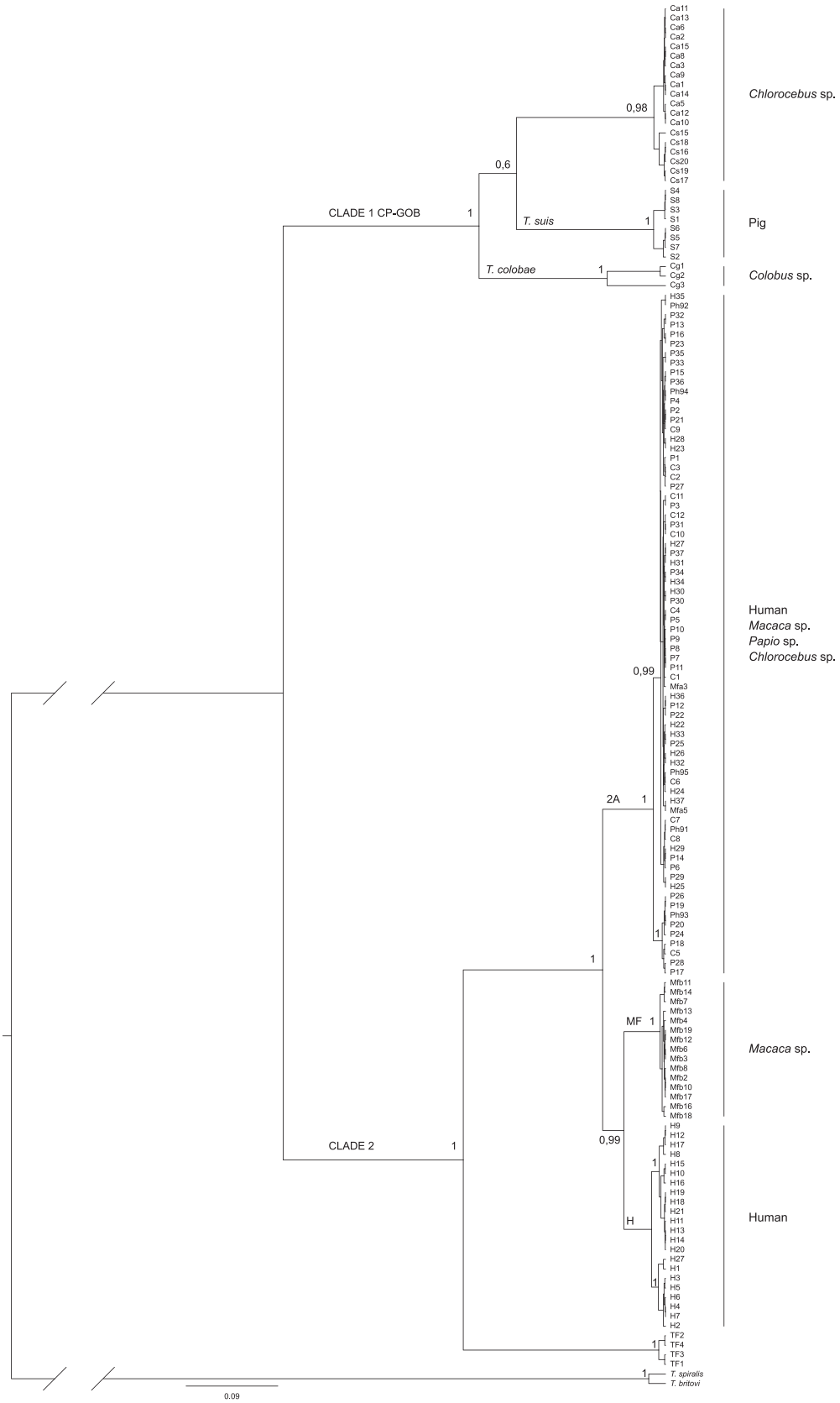

Fig. 1. Bayesian tree showing the relationships among *Trichuris* species, based on the analysis of the DatasetrrnL, including sequences used as outgroup (*Trichinella spiralis* and *T. britovi*), with indications on host affiliation and assignment to clades and subclades, following the nomenclature used in previous papers (Ravasi et al., 2012; Callejón et al., 2013; Cavallero et al., 2015). *Trichuris* spp. with host affiliation and sequence codification according to Table 1 are indicated at the end of branches. Posterior probability values are reported and nodes with less than 0.95pp were collapsed; number of expected mutation per site is indicated by scale bar.

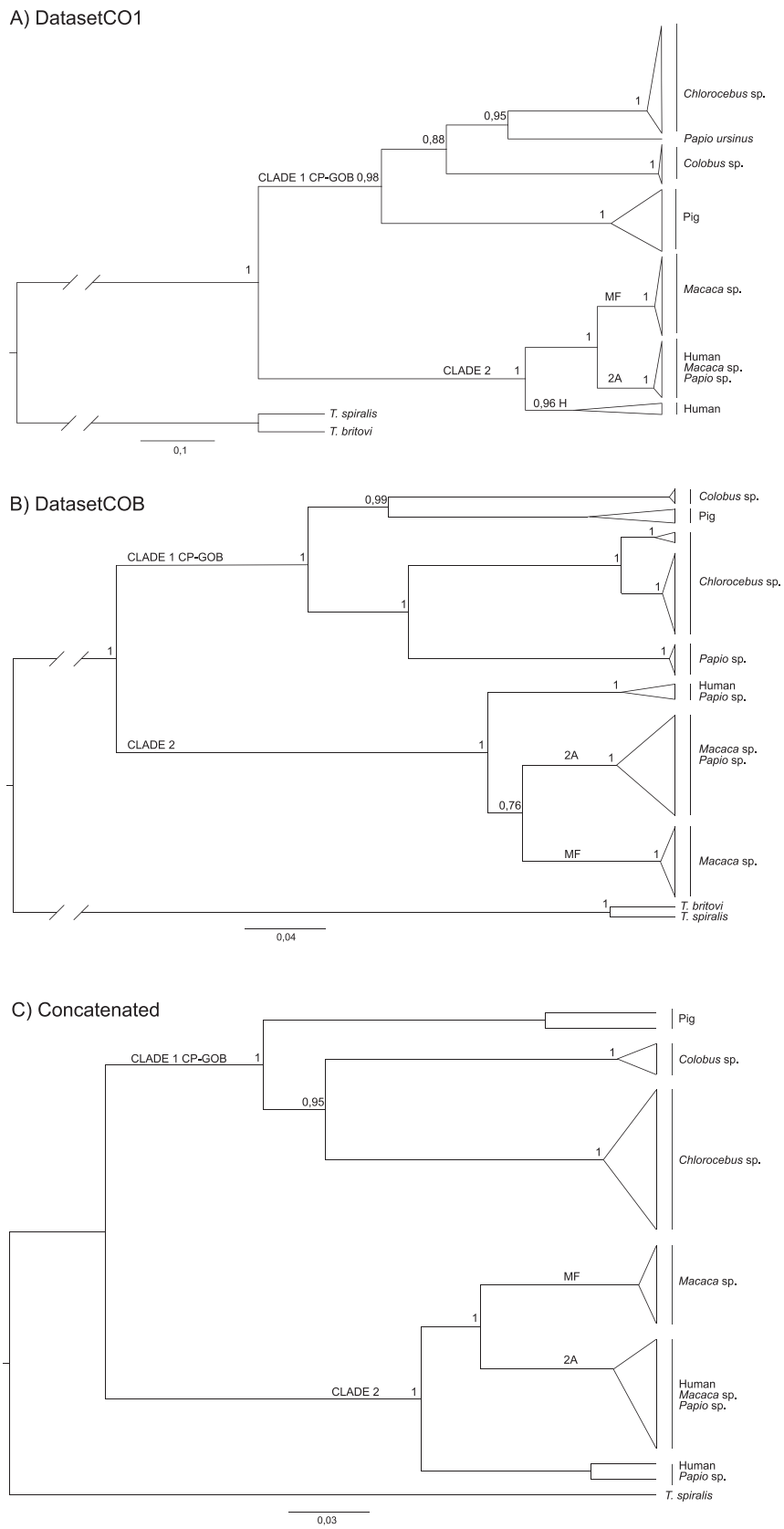

Fig. 2. Bayesian tree showing the relationships among *Trichuris* species, based on the analysis of the DatasetCO1(A), DatasetCOB (B) and concatenated (C) including sequences used as outgroup, with indications on host affiliation and assignment to clades and subclades, following the nomenclature used for DatasetrrnL and in previous papers (Ravasi et al., 2012; Callejón et al., 2013; Cavallero et al., 2015). Posterior probability values are reported at nodes; number of expected mutation per site is indicated by scale bar.

followed by a dispersal along Asia where a host-shift to pig may have occurred, giving rise to speciation events and to *T. suis*. The occurrence of ancestral primates and swine in the same areas during the past, probably in the late Pleistocene, may have favored the switching of *T. trichiura* from primates to swine. The close relationship between the two mentioned species is also documented by the ability of *T. suis* to both infect and mature in humans (Nissen et al., 2012; Phosuk et al., 2018).

We found that all *Trichuris* from the genus *Chlorocebus* clustered together, with the exception of some DNA sequences retrieved from GenBank, which clustered in Clade 2 in DatasetrrnL. Bayesian analysis showed two internal branches probably reflecting two geographically and/or host-affiliated entities: one infecting *C. aethiops* from central-eastern Africa, and the other infecting *C. sabaeus*, which is distributed in central-western Africa. The inclusion of *Trichuris* from green monkeys in the same evolutionary clade as *T. suis* and *T. colobae* may also relate to similarities or convergence in protein evolution, which could be suggested because of the shared polymorphisms among these taxa. It has been observed in other parasitic nematodes that such unusual patterns in phylogenetic scenarios based on mitochondrial DNA sequences may be the result of the retention of ancestral polymorphisms as well as of backward mutations (Cavallero et al., 2013; Betson et al., 2014).

Despite of this, *Trichuris* of human origin were observed only in Clade 2 in the present investigation; this is in line with all previous studies using mitochondrial markers whereas studies applying nuclear ribosomal markers also observed human *Trichuris* in the so-called Clade 1CP-GOB (Ravasi et al., 2012; Cavallero et al., 2015). Phylogenetic signals obtained by nuclear and mitochondrial markers may be non-concordant, as reflection of potentially different evolutionary rates of genomic regions, and correspondence of clades could be difficult to infer. Their combined use may help to resolve the relationships among geographic populations and species within the genus *Trichuris* (Callejón et al., 2013), as well as a dense taxon sampling may help to provide a reliable phylogeny at species level (Omland et al., 1999; Agnarsson and May-Collado, 2008).

Clade 2 included *Trichuris* sp. from *M. fuscata*, analogous to subclade MF reported by Cavallero et al. (2015) and related to *T. trichiura* and other species of primates. Interestingly, these Japanese macaques hosted at the Bioparco Zoological Garden of Rome showed to harbor two potentially distinct entities of *Trichuris*, varying across 51 out of 341 nt in *cox*1. This might suggest two different sources of infection and that two *Trichuris* taxa infect Japanese macaques; one potentially able also to infect humans. However, unexpected transmission events may occur in captivity as observed for *Ascaris suum* in *Pan troglodytes* (Nejsum et al., 2010), which needs to be considered as well.

The level of genetic variation in mtDNA genes between related species has been found to be between 10%–20%, not exceeding 6% between a pair of individuals that were clearly members of the same interbreeding population, as observed in the brown stomach worm, *Ostertagia ostertagi* (Blouin et al., 1998; Blouin, 2002). Generally, genetic distance values below 2%–6% are thought to reflect the existence of cryptic species, while values exceeding 10% are suggestive of different morphospecies. The genetic diversity previously reported for human *T. trichiura* from different regions (South America, Asia and Africa) has amounted to about 20%, and values up to 30% were observed in comparison to *T. suis* (Hawash et al., 2016). Similarly, genetic distances between *T. rhinopiptheroxella* and other *Trichuris* from humans, *Trachypithecus francoisi* and *Papio* spp. were around 27% (Wang et al., 2018).

Likewise, the genetic diversity observed in the present study for already described *Trichuris* species infecting other mammalian hosts ranged from 0.20%–0.22% between very closely related species (e.g. *T. muris* vs *T. arvicolae* - data not shown) and up to 20% between *T. trichiura* and *T. suis* (S1, Tables 2 and 3).

Our observations suggest that more than one *Trichuris* taxon is able to infect primates. However, as there is no clear definition as to what extent nematodes should differ genetically to be considered separate species, such estimations should be carefully considered only as indicative, given the small amount of data analyzed and their origin from captive animals.

In the present study, phylogenetic inferences obtained using three different mitochondrial markers, both as separated and concatenated datasets, are consistent with the evolutionary distance data. Bayesian analysis supports the existence of two main lineages as previously suggested (Doležalová et al., 2015). Within these two clades, five taxonomic entities able to infect primates have been detected: some showing strict host specificity, as for grivets and green monkeys; others infecting more than one species, as evidenced by the fact that they are shared by several primates, namely humans, baboons, and macaques.

The obtained tree topologies based on *Trichuris* from NHPs reflected the suggested phylogeny of the primate host (Perelman et al., 2011; Finstermeier et al., 2013). The two-clade branching pattern of *Trichuris* appears to mirror that observed in their respective hosts belonging to the Cercopithecidae; i.e., one clade including Papionini (*Macaca* spp. and *Papio* spp.) and the other including Cercopithecini (*Chlorocebus* spp.).

Putative complexes of cryptic *Trichuris* species with some degree of host preference were observed, since no clear clustering, both of human and NHPs derived *Trichuris* were obtained by any of the above-mentioned phylogenetic studies. Considering that species have recently been described in NHPs [*T. colobae* in *Colobus guereza* (Cutillas et al., 2014) and *T. ursinus* in *P. ursinus*, (Callejón et al., 2017)], *T. trichiura* may be considered as a species complex with several sibling/cryptic species. However, further studies including analysis of *Trichuris* samples from different geographical locations and host species are needed to explore this hypothesis, using multispecies coalescent model to test hypotheses of separate species (Edwards et al., 2016) and population genetics approaches.

5. Conclusions

Our analyses suggest a complex scenario with multiple *Trichuris* species infecting primates and that some of these may be shared between humans and NHPs, with clear implications on both the systematics of *Trichuris* and the control of whipworm infections in both humans and NHPs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.06.019.

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