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How many species of whipworms do we share? Whipworms from man and other primates form two phylogenetic lineages

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Abstract: The whipworms, i.e. parasitic nematodes of the genus *Trichuris* Roederer, 1761, infect a variety of mammals. Apparently low diversity of primate-infecting species of *Trichuris* strongly contrasts with the high number of species described in other mammalian hosts. The present study addresses the diversity of whipworms in captive and free-ranging primates and humans by analysing nuclear (18S rRNA, ITS2) and mitochondrial (*cox1*) DNA. Phylogenetic analyses revealed that primate whipworms form two independent lineages: (i) the *Trichuris trichiura* (Linnaeus, 1771) clade comprised of genetically almost identical whipworms from human and other primates, which suggests the ability of *T. trichiura* to infect a broader range of primates; (ii) a clade containing primarily *Trichuris suis* Schrank, 1788, where isolates from human and various primates formed a sister group to isolates from pigs; the former isolates thus may represent of more species of *Trichuris* in primates including humans. The analysis of *cox1* has shown the polyphyly of the genera *Trichuris* and *Capillaria*, Zeder, 1800. High sequence similarity of the *T. trichiura* isolates from humans and other primates suggests their zoonotic potential, although the extent of transmission between human and other non-human primates remains questionable and requires further study.

Keywords: *Trichuris*, phylogeny, diversity, zoonotic potential, humans

This article contains supporting information (Figs. S1–S5) online at http://folia.paru.cas.cz/suppl/2015-62-063.pdf

The nematode genus *Trichuris* Roederer, 1761 includes more than 60 species that parasitise various mammalian species from the orders Artiodactyla, Carnivora, Didelphimorphia, Lagomorpha, Peramelemorphia, Primates and Rodentia (Anderson 2000). For decades, taxonomic studies almost exclusively focused on whipworm species from ruminants or rodents (e.g. Knight 1983, Robles 2011, Torres et al. 2011), probably due to better accessibility to material from these hosts.

Whipworm infections are common in many species of free-ranging primates in Africa (e.g. Ashford et al. 2000, Kalema-Zikusoka et al. 2005), America (Michaud et al.

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2003, Phillips et al. 2004) and Asia (Mul et al. 2007, Labes et al. 2010) as well as those in captivity (Melfi and Poyser 2007, Lim et al. 2008). *Trichuris lemuris* Rudolphi, 1819 and *Trichuris cynocephalus* Khera, 1951 are together with *Trichuris trichiura* (Linnaeus, 1771) species described from primates (Khera 1951, Chabaud et al. 1964). Since infections with whipworms in primates were usually diagnosed as those caused by *T. trichiura* (see Ooi et al. 1993, Reichard et al. 2008, Lee et al. 2010), several authors have expected zoonotic cross-transmission between non-human primates and humans (Munene et al. 1998, Chapman et al. 2006).

Such an unusually broad host range of *T. trichiura* raises the questions of host specificity and possibility of underestimated diversity of species in non-human primates. The low diversity of species of *Trichuris* in primates strongly contrasts with the high number of those found in other mammalian hosts. The assemblage of species of *Trichuris* infecting rodent hosts is probably the most diversified one, with 21 species reported from rodents of nine families in North and South America (Robles 2011). Another 11 species of *Trichuris* parasitise four families of ruminants (e.g. Knight 1983, Cutillas et al. 2004).

The exact species determination of *Trichuris* spp. based solely on egg morphology is virtually impossible (Špakulová 1994) and adult worms are difficult to obtain from primates. Thus, molecular taxonomy offers a powerful approach for the evaluation of diversity of trichurid species in these hosts. During the past decades, nuclear (ITS1-5.8S-ITS2) and mitochondrial (*cox1*) DNA have been used for species identification, as well as for phylogenetic analyses of species of *Trichuris* from rodents, pigs and cattle (Cutillas et al. 2004, 2009, Callejón et al. 2009, 2012).

Recently, the analyses of sequences of the *cox1* gene of mitochondrial DNA have confirmed that *Trichuris suis* Schrank, 1788 and *T. trichiura* represent two distinct species (Liu et al. 2012). Interestingly, studies using ITS2 sequence have also claimed that isolates of *T. trichiura* from humans and non-human primates form two distinct clades, although the interpretations of two studies were different. Whereas Nissen et al. (2012) found in humans two types of clones of *T. trichiura* (one similar to *T. trichiura* and another similar to *T. suis*) and heterozygotes that have both types of rDNA, Ravasi et al. (2012) concluded that these findings were consequence of cross-contamination of DNA and showed on their own isolates the suggested existence of distinct species of *Trichuris* sp. in human and non-human primates.

The present study contributes to a better understanding of the diversity of species of *Trichuris* in humans and non-human primates using phylogenetic analyses combining both nuclear and mitochondrial genes. Moreover, the hypothesised existence of more species of *Trichuris* in primates opens the possibility to revise the zoonotic potential and host specificity of *T. trichiura* and other, putative new species of whipworms.

MATERIALS AND METHODS

Study sites and sampled hosts

Faecal samples containing the eggs of whipworms (detected by coproscopic techniques, see below) were obtained from several captive primate species including chimpanzee, *Pan troglodytes* Blumenbach, yellow-cheeked gibbon, *Nomascus gabriellae* Thomas, olive baboon, *Papio anubis* Lesson, and gelada baboon, *Theropithecus gelada* Rüppell, lion-tailed macaque, *Macaca silenus* Linnaeus, green monkey, *Chlorocebus sabaeus* Linnaeus and rhesus macaque, *Macaca mulatta* Zimmermann. Adult whipworms were acquired during necropsy from hamadryas baboon, *Papio hamadryas* Linnaeus, long-tailed macaque, *Macaca fascicularis* Raffles, and eastern black-and-white colobus monkey, *Colobus guereza kikuyuensis* Lönnberg; nematodes from the latter host were in the meantime described as *Trichuris colobae* Cutillas, Oliveros et Callejón, 2014.

Faecal samples from free-ranging primates were acquired from two sites: one isolate from a wild chimpanzee residing in the Cantanhez National Park, Guinea-Bissau; six samples from a chimpanzee, vervet monkeys, *Chlorocebus aethiops pygerythrus* Cuvier, and black-and-white colobus monkey, *Colobus guereza guereza* Rüppell, living in the Rubondo Island National Park, Tanzania. Finally, a single adult female of *Trichuris* was obtained from an anonymous Czech researcher.

In addition, eggs of whipworms isolated from domestic pig, *Sus scrofa* Linnaeus, domestic dog, *Canis lupus* Linnaeus, and Bactrian camel, *Camelus bactrianus* Linnaeus, were included into the study (summary of all isolates in Table 1).

Sample collection and preparation

Faecal samples from captive animals were collected immediately after defecation and preserved in 20 ml of 10% formalin (2–5 g) and 5 ml of 96% ethanol (~2 g), the samples from free-ranging chimpanzees, colobus and vervet monkeys were collected as described in Petrášová et al. (2010) and Sá et al. (2013). The adult whipworms were acquired by colonoscopy (*Homo sapiens*, isolate 1) or necropsy of hosts (*Macaca fascicularis* – isolate 9, *Papio hamadryas* – isolate 7, *Colobus guereza kikuyuensis* – isolate 12) and also preserved in 96% ethanol.

The flotation technique with modified Sheather's solution (Sheather 1923) was used for initial coproscopic analysis of formalin-preserved samples. In cases where whipworm eggs were present (Fig. 1), ethanol preserved samples were processed by flotation technique as well. The obtained eggs were collected from the surface of the flotation solution using a horizontal wire loop and transferred into a 10 ml vial with 8 ml of phosphate buffered saline (PBS). Thereafter, the vial was centrifuged 2 min at ~320 × *g* and the pellet containing eggs was suspended in 1 ml of PBS; 40 µl was pipetted onto a microscopic slide using a dimple.

Individual eggs were sucked up by a thin glass micropipette (normally used for embryo transfers) equipped with a short silicone hose having an inner diameter of 2 mm and a node on one end. The eggs (minimum 30) were transferred into 0.5 ml of PBS in a 2 ml micro tube. Prior to DNA extraction, the egg shells were disrupted with glass beads (0.5 and 1 mm) using a BeadBeater

Table 1. The locality, source of DNA and GenBank accession numbers of particular genes of whipworm isolates acquired from non-human primates and humans. The extended data set includes isolates from a domestic pig, dog and Bactrian camel. The whipworm isolates from colobus monkeys (13, 14 and 15) and chimpanzees (3, 4) were identical in the 18S rRNA gene sequences with that from gelada baboon.

Isolate	Host	Locality	Source of DNA	*cox1*	18S'rDNA	ITS2
1	*Homo sapiens*	Brno, CZ	adult	JF690962	JF690953	JF690940
2	*Pan troglodytes* Blumenbach	Antwerp Zoo, NL	eggs	-	-	JF690948
3	*Pan troglodytes*	Rubondo NP, EAT	eggs	-	+	-
4	*Pan troglodytes*	Cantanhez NP, GW	eggs	-	+	-
5	*Nomascus gabriellae* Thomas	Bratislava Zoo, SK	eggs	-	-	JF690947
6	*Papio anubis* Lesson	Brno Zoo, CZ	eggs	JF690964	JF690955	JF690942
7	*Papio hamadryas* Linnaeus	Liberec Zoo, CZ	adult	JF690963	JF690954	JF690941
8	*Theropithecus gelada* Rüppell	Brno Zoo, CZ	eggs	JF690965	JF690956	JF690943
9	*Macaca fascicularis* Raffles	Konárovice, CZ	adult	JF690967	JF690958	JF690946
10	*Macaca mulatta* Zimmermann	Konárovice, CZ	eggs	-	JX049339	-
11	*Macaca silenus* Linnaeus	Liberec Zoo, CZ	eggs	JF690966	JF690957	JF690945
12	*Colobus guereza kikuyuensis* Lönnberg	Fuengirola Zoo, E	adult	JF690968	JF690959	FM991956*
13	*Colobus guereza* Rüppell	Rubondo NP, EAT	eggs	-	+	-
14	*Colobus guereza*	Rubondo NP, EAT	eggs	-	+	-
15	*Colobus guereza*	Rubondo NP, EAT	eggs	-	+	-
16	*Chlorocebus sabaeus* Linnaeus	Liberec Zoo, CZ	eggs	-	JX049338	JF690944
17	*Chlorocebus aethiops pygerythrus* Cuvier	Rubondo NP, EAT	eggs	-	-	JF690949
18	*Chlorocebus aethiops pygerythrus*	Rubondo NP, EAT	eggs	-	-	JF690950
19	*Sus scrofa* Linnaeus	Okoč, SK	eggs	JF690969	JF690960	JF690951
20	*Canis lupus* Linnaeus	Brno, CZ	eggs	-	JX049340	-
21	*Camelus bactrianus* Linnaeus	Brno Zoo, CZ	eggs	JF690970	JF690961	JF690952

Amplified genes of particular isolates have sign '+' or GenBank Accession Number, not amplified genes are signed by '-'. CZ – Czech Republic, E – pain, EAT – Tanzania, GW – Guinea-Bissau, NL – Netherlands, NP – National Park, SK – Slovak Republic. * Cutillas et al. (2009).

(Biospec Products Inc., Bartlesville, Oklahoma, USA) for 10 min at a shaking frequency of 2 100 oscillations/min.

The adult whipworms were washed 5× in PBS, disintegrated by micro-pestles and washed 5× in PBS, followed by centrifugation for 5 min at ~16 000× *g*. After the last centrifugation, the supernatant was removed and the pellet was prepared for DNA extraction.

DNA extraction

Before DNA extraction, 800 µl of NET buffer pH 8.0 (4 M NaCl, 0.5 M EDTA and 1 M TRIS), 240 µl of 30% N-lauroylsarcosine sodium salt solution (Sigma-Aldrich, Praha, Czech Republic) and 30 µl of proteinase K (Chemos CZ, Praha, Czech Republic) were added to the prepared samples. The homogenate was incubated in a dry bath at 56 °C for 15 h and subsequently extracted using the phenol-chloroform method based on the protocol outlined by Sambrook and Russell (2006). The phenol-chloroform extraction yielded DNA at concentrations of 10–15 ng/µl from eggs and 30–130 ng/µl from adults.

PCR amplification and sequencing

The partial ITS2 and 18S of ribosomal RNA (rDNA) and *cox1* of mitochondrial DNA (mtDNA) were amplified by PCR using a Biometra thermocycler (Biometra, Göttingen, Germany). For ITS2 (600 bp), the forward primer Proteo1 (5'-CGGTGGATCACTCGGCTC-3'; Škeříková et al. 2004) and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990) were used with the following PCR conditions: an initial denaturation at 96 °C for 5 min and then 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Genus-specific primers were designed to amplify a 550 bp fragment of 18S rDNA: forward primer TF (5'-GATGTCCACTTGGATAACTA-3') and reverse primer TR (5'-GACGGAACGACTCCTGCTTA-3'). PCR was performed using the following conditions: an initial denaturation at 96 °C for 3 min and then 30 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Finally, a 419 bp fragment of the *cox1* gene was amplified using the forward primer L6625 (5'-TTYTGRTTYTTYGGNCAYCC-3') and reverse primer H7005 (5'-ACTACGTAGTAGGTATCATG-3'), which were together with PCR conditions modified by Callejón et al. (2009). Each PCR mixture included: 12.5 µl of commercial Combi PPP Master Mix (Top-Bio, Praha, Czech Republic), 9.5 µl of PCR water, 1 µl of each primer mix (0.01 mM each) and 1 µl of template DNA. The PCR products were checked on an ethidium bromide-stained 1% agarose gel. PCR products were purified from the agarose gel using the QuickClean 5M Gel Extraction Kit (GenScript USA, Inc., Piscataway Township, New Jersey, USA) according to manufacturer's protocol and sequenced at Macrogen Inc. (Seoul, Korea). The nucleotide sequences obtained in our study have been deposited in GenBank under accession numbers FJ690940–FJ690970 and JX049338–JX049340.

To verify the possible presence of paralogs in the genome of species of *Trichuris*, primers according to Logan et al. (1992) were used to amplify the complete sequence of the 5.8S ribosomal RNA gene (rDNA) surrounded by partial sequences of internal transcribed spacers 1 and 2. For this purpose, we have randomly chosen the isolate of *Trichuris* sp. from *Papio hamadryas* residing in the Liberec Zoo. Obtained PCR products were cloned and six were sequenced in both directions. No paralogs were detected (data not shown).

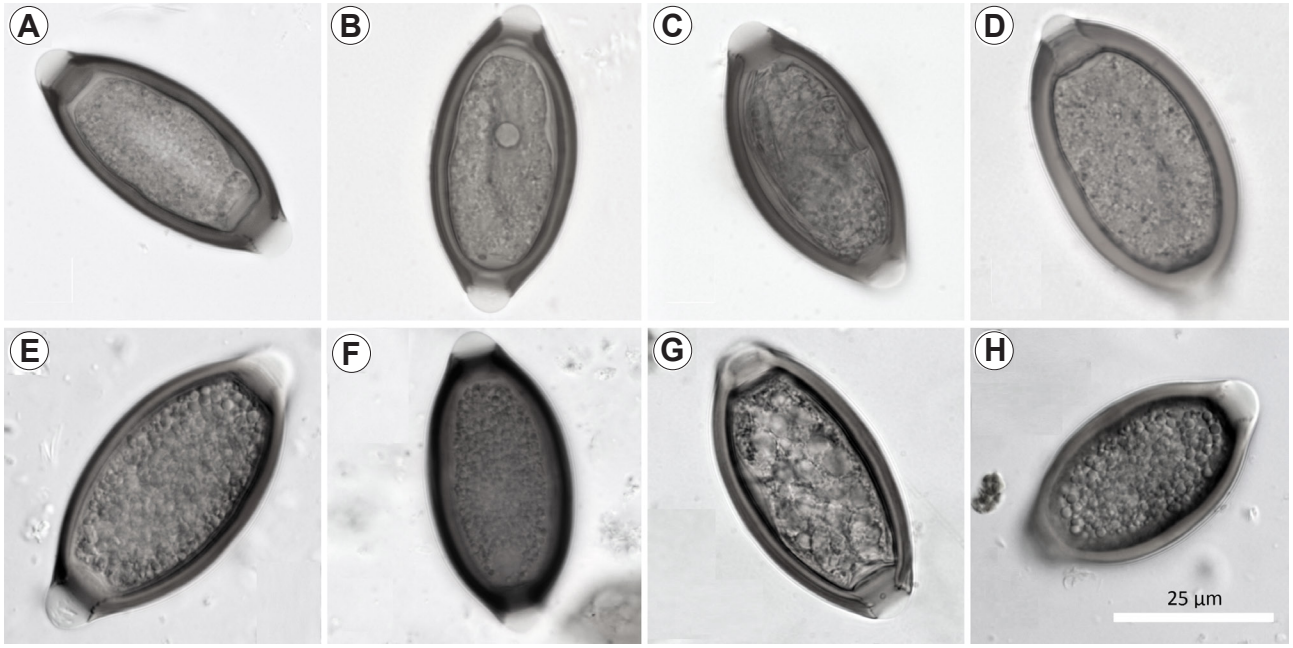

Fig. 1A–H. The variability of the eggs of *Trichuris* spp. retrieved from the faeces of primates and a domestic pig; Nomarski interference contrast with an Olympus AX70 microscope; all at the same scale. **A** – *Trichuris trichiura* (Linnaeus, 1771) from human patient (isolate1); **B** – *Trichuris* sp. from *Macaca fascicularis* (isolate 9); **C** – *Trichuris* sp. from *Chlorocebus aethiops pygerythrus* (isolate 18); **D** – *Trichuris* sp. from *Chlorocebus aethiops pygerythrus* (isolate 17); **E** – *Trichuris suis* from domestic pig (isolate 19); **F** – *Trichuris* sp. from *Macaca silenus* (isolate 11); **G** – *Trichuris* sp. from *Pan troglodytes* (isolate 4); **H** – *Trichuris* sp. from *Chlorocebus sabaeus* (isolate 16).

Phylogenetic analyses

We used sequences from three different genomic regions to infer phylogenetic relationships among species of *Trichuris* and related taxa, namely nuclear ITS2 region of the rRNA gene, part of the small subunit rRNA gene (18S rDNA) and the mitochondrial gene coding for *cox1*. Nuclear genes were aligned using their nucleotide sequence whereas inferred amino acid sequence data were used for *cox1*.

Thus, we aligned 77 ITS2 GenBank™ sequences of *Trichuris* spp. from human and animal hosts with an additional 13 newly obtained sequences from humans, non-human primates, pig and camel (Table 1) using the program Kalign (Lassmann and Sonnhammer 2005). The same program was used to align 38 18S rDNA genes of species of the genus *Trichuris,* of which 17 were newly obtained. Gaps and ambiguously aligned positions were removed using BioEdit (Hall 1999). Due to high number of gaps, the alignments of ITS2 and partial 18S rDNA gene sequences were also constructed using webPRANK (http://www.ebi.ac.uk/goldman-srv/webprank/) probabilistic approach and the alignments were edited by Gblocks (Castresana 2000, Talavera and Castresana 2007) in the frame of SEAVIEW program (Gouy et al. 2010).

Phylogenetic trees were computed by maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) using PhyML (Guindon and Gascuel 2003), PAUP 4b10 (Swofford 2002) and PhyloBayes 3.2 (Ronquist et al. 2012), respectively. ML trees were calculated under the gamma corrected GTR evolutionary model (GTR + G). Bootstrap support of ML and MP trees was inferred from 1 000 replicates. Bayesian topologies and posterior probabilities (PP) were assessed under the gammacorrected GTR substitutional matrix (GTR + G) using MrBayes 3.2 (Ronquist et al. 2012). Two independent Monte-Carlo Markov chains were allowed to run (under the default settings) for three million generations, of which the first 5×10^5 were omitted from PP (and topology) reconstruction. The ITS2 tree was rooted to *Trichuris skrjabini* Baskakov, 1924 (AJ489248 – outgroup); the 18S tree was rooted to *Trichuris muris* (Schrank, 1788) (AF036637 – outgroup). The ML trees were computed using alignments constructed by webPRANK only (see Figs. S1, S2).

We also constructed two trees (for intraspecific and interspecific variability) inferred from *cox1* amino acid datasets by Neighbor Joining (NJ), MP and ML methods using AsaturA (Van de Peer et al. 2002; AsaturA is a program designed to deal with amino acid saturation), PAUP 4b10 (Swofford 2002) and PhyML (Guindon and Gascuel 2003) programs, respectively. AsaturA was used to avoid phylogenetic artifacts, because sequences HQ204213, HQ204211, HQ204212, AJ288166, AJ288168 and AJ288169, and new sequences from isolates 9 and 11 constituted long branches in the tree.

For testing intraspecific variability the dataset comprised 44 *cox1* sequences that are proposed to belong to the genus *Trichuris* (including 9 new sequences). For testing interspecific variability, this dataset was enriched by sequences of species of the related genera *Capillaria* Zeder, 1800 (10 sequences) and *Anatrichosoma* Swift, Boots et Miller, 1922 (1 sequence). Sequences were aligned by Kalign (Lassmann and Sonnhammer 2005), and the alignment was manually verified and edited in BioEdit (Hall 1999); NJ and ML trees were computed using the LG model that was gamma corrected in the case of ML (LG + G + I). Bootstrap supports were computed from 1 000 replicates.

Furthermore, a combined dataset of the three gene regions (for which data were available) was used to estimate the deep phy-

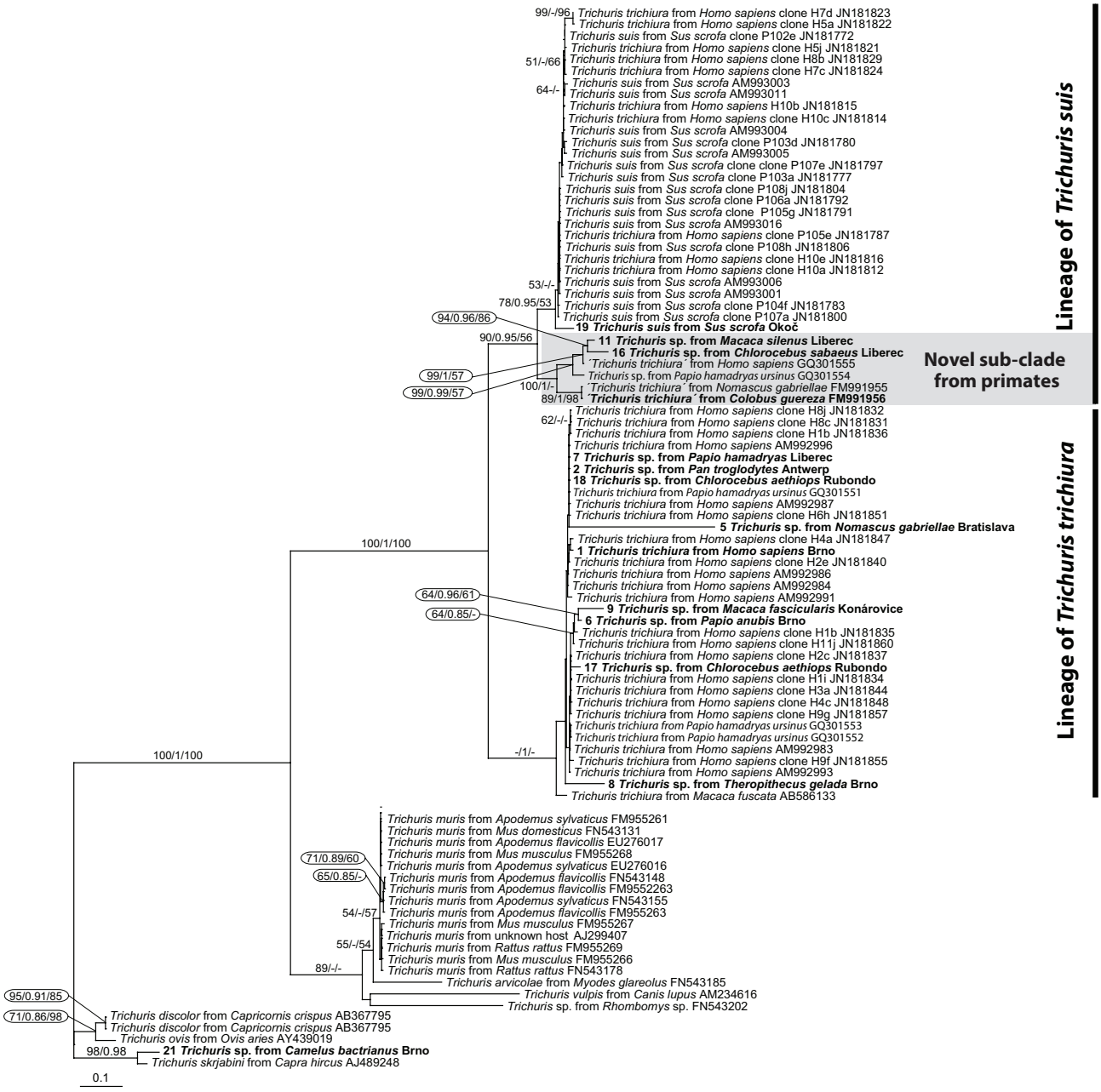

Fig. 2. Maximum likelihood phylogenetic tree as inferred from ITS2 sequences. The tree was computed using the GTR model with discrete gamma distribution in four categories. Numbers above branches indicate ML bootstrap support (1 000 replicates)/Bayesian posterior probabilities (PhyloBayes; see Methods for details)/MP bootstrap support (1 000 replicates). Sequences newly reported in this study are bold typed. The three basic clades of genus *Trichuris* are highlighted. Tree was rooted to *Trichuris skrjabini* Baskakov, 1924 (AJ489248).

logenetic positions of *Trichuris* isolates. For this purpose we used *Trichuris* isolates from *Sus scrofa* (isolate 19), *Camelus bactrianus* (isolate 21), *Colobus guereza* (isolate 12), *Macaca silenus* (isolate 11), *M. fascicularis* (isolate 9), *Papio anubis* (isolate 6), *P. hamadryas* (isolate 7), *Theropithecus gelada* (isolate 8) and *Homo sapiens* (isolate 1). These were studied together with *Trichuris trichiura* (2 samples), *T. suis* (4 samples), *T. arvicolae* Feliu, Morand et Hugot, 2000 (1 sample), *T. muris* (1 sample), *T. ovis* (Abildgaard, 1795) (1 sample), *T. discolor* (von Linstow, 1906) (1 sample) and *T. skrjabini* (1 sample) sequences deposited in the GenBank database. The nucleotide alignment of individual gene was constructed separately using Kalign (Lassmann and Sonnhammer 2005); inferred *cox1* amino acid sequences were aligned in BioEdit.

All three gene regions were consequently concatenated into a single dataset. ML and BI trees were computed using RAxML 8.1a (Stamatakis 2014) and MrBayes 3.2 (Ronquist et al. 2012), respectively. The dataset was analysed as partitioned (i.e. the model parameters were estimated separately for each respective gene) under the gamma-corrected GTR (GTR + G) (ML and BI) and HKY models (BI). Bootstrap supports of ML tree were computed from 1 000 replicates. In BI, two independent chains were run for 3×10^6 generations and posterior probabilities (as a mean of branching support) were estimated after first 1×10^5 genera-

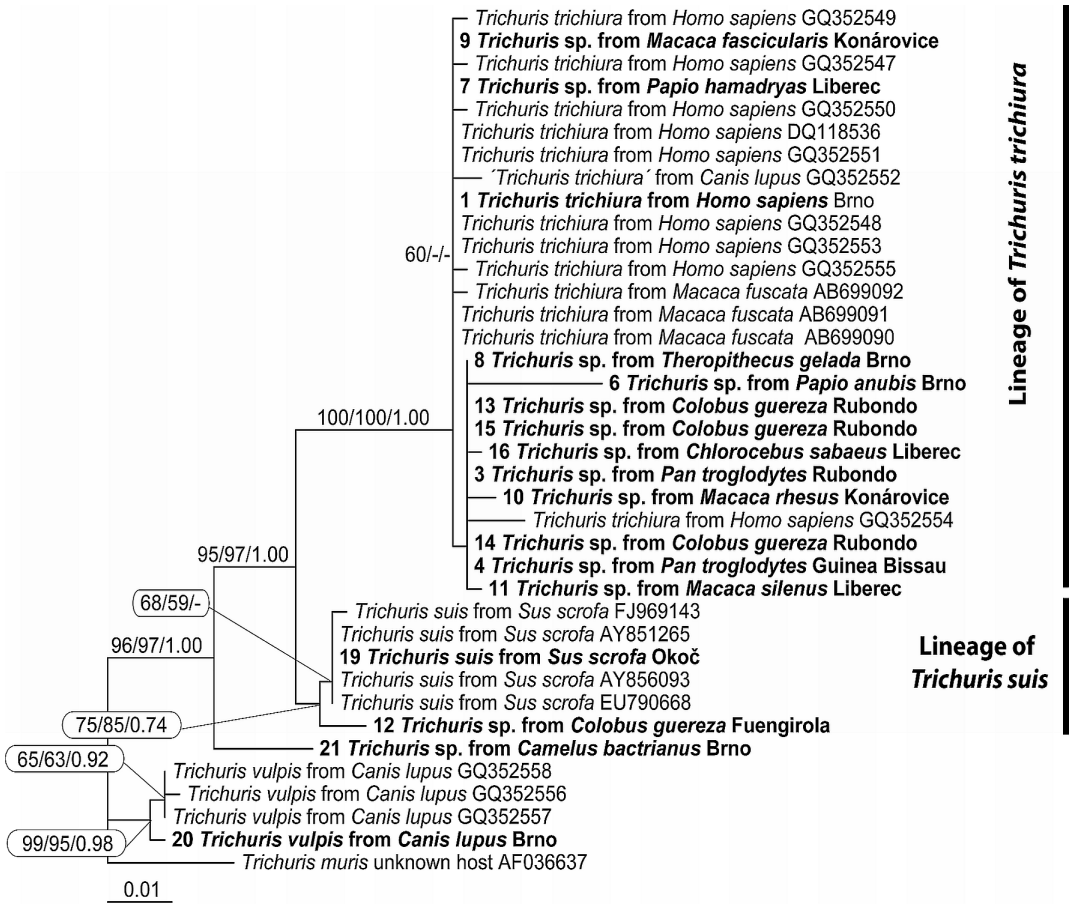

Fig. 3. Maximum parsimony phylogenetic tree as inferred from partial 18S rRNA gene sequences. Numbers above branches indicate MP bootstrap support (1 000 replicates)/ML bootstrap support (1 000 replicates)/Bayesian posterior probabilities (see Methods for details). Sequences newly reported in this study are bold typed and basic clades are highlighted. Tree was rooted with *Trichuris muris* (Schrank, 1788) (AF036367).

tions were discarded as a 'burnin'. The unrooted combined tree is shown (Fig. S3).

RESULTS

The described method of DNA isolation from whipworm eggs proved to be rather successful as we were able to retrieve DNA from almost 90% of the samples of eggs collected (negative data not shown). The lengths of sequences were 550–620 bp for ITS2, 669–750 bp for 18S rDNA, and 390–430 bp for *cox1*. Due to a low amount of DNA from the eggs in some isolates, we obtained 13 ITS2, 17 18S rDNA and 9 *cox1* amplicons. The 18S and ITS2 alignments were over 518 and 327 positions, respectively, whereas the *cox1* alignment was comprised of 124 amino acids. These datasets were analysed separately and also in a concatenated alignment. This alignment contained 525 18S rDNA positions, 354 ITS2 positions and 372 *cox1* positions (1 251 positions in total).

The primary Kalign alignment of ITS2 was 1 425 nucleotides long. Manual editing (deleting gaps and ambiguously aligned regions) produced alignment of 327 nucleotides, alternative Gblock selection resulted in alignment 383 nucleotides long. When the webPRANK approach was applied to the dataset, it produced primary alignment consisting of 1 465 nucleotides; when the Gblock was used to delete gaps (primary alignment contained positions unusable for phylogenetic analysis, mainly due to the occurrence of gaps), the resulting alignment contained 276 nucleotides. However, in spite of the mentioned differences, all alignments showed almost identical but weakly supported topologies (Figs. 2, S1). The primary alignment of 18S rDNA consisted of 555 nucleotides, which was reduced by manual editing to 518 nucleotides long alignment. The primary alignment constructed by webPRANK was 545 nucleotides long and for tree computation, it was reduced using Gblock to 523 nucleotides (primary alignment contained positions unusable for phylogenetic analysis, mainly due to the occurrence of gaps in a fraction over 80%). Trees inferred from the mentioned alignments do not display substantial differences in topology.

ML analysis based on ITS2 revealed three basic lineages within the genus *Trichuris* (Fig. 2). The first lineage was mostly composed of whipworms from pigs (classified as *T. suis*; lineage of *T. suis*), with a separate subclade containing isolates from man and various primates (a novel subclade from primates; see Fig. 2).

The second lineage contained sequences of *Trichuris* classified as *T. trichiura* (Fig. 2) isolated from humans and non-human Old World primates. This clade contained a large group of almost identical whipworm se-

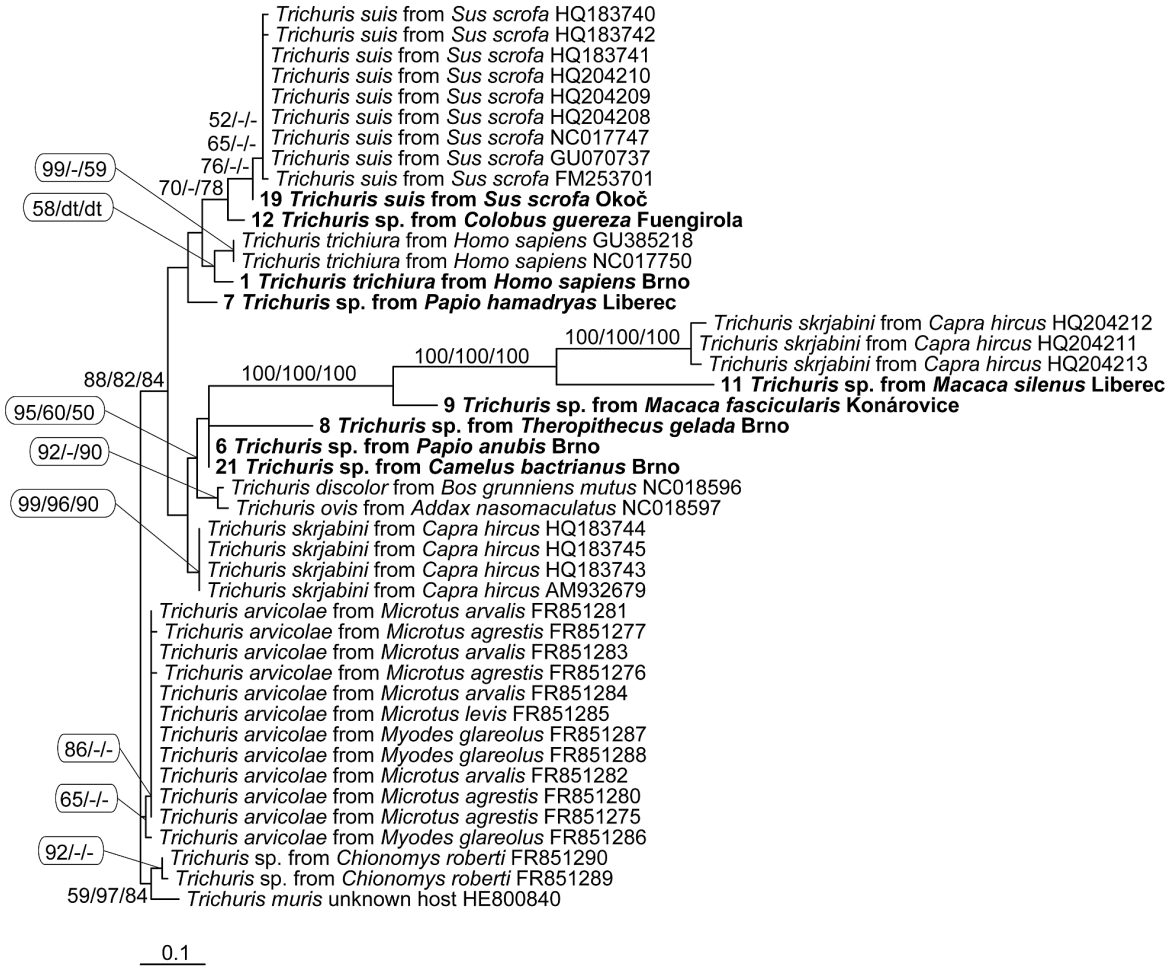

Fig. 4. Unrooted maximum parsimony *cox1* tree of the genus *Trichuris* Roederer, 1761 as inferred from partial *cox1* amino acid sequences. Numbers above branches indicate MP bootstrap support (1 000 replicates)/ML bootstrap support (1 000 replicates)/NJ bootstrap support (1 000 replicates). Sequences newly reported in this study are bold typed.

quences from humans and primates and also six relatively distant sequences from *Macaca fascicularis* (isolate 9), *M. fuscata* Blyth (AB586133), *Papio anubis* (isolate 6), *Theropithecus gelada* (isolate 8), *Chlorocebus aethiops pygerythrus* (isolate 17) and *Nomascus gabriellae* (isolate 5).

The third lineage comprised whipworms from rodents and carnivores containing several morphologically and biologically distinguishable species [*T. muris*, *T. arvicolae* and *T. vulpis* (Frölich, 1789)].

The tree based on partial 18S rDNA sequences (Fig. 3) contained a substantially lower number of sequences. However, isolates from humans and non-human primates again formed two distinct clades. The first clade was composed of genetically almost identical isolates classified as *Trichuris trichiura*, including sequences from *Macaca silenus* (isolate 11), *Chlorocebus sabaeus* (isolate 16) and one isolate from dog (a sequence of ambiguous origin), whereas the isolate from *Colobus guereza kikuyuensis* (isolate 12) is placed at the root of the second *T. suis* clade. Both clades of *T. trichiura* and *T. suis* formed sister groups, similar to analyses based on ITS2 data. The *Trichuris* specimen from camel formed a basal clade to both of the mentioned groups. The last clade comprised *T. vulpis* and *T. muris* isolates (Fig. 3). No substantial differences were found between trees inferred from manually edited alignments (shown in Figs. 2, 3) and those obtained using probabilistic webPRANK and edited by Gblocs to delete gaps from the alignment (see Figs. S1, S2). Composition of main clades is identical, only some particular positions of closely related isolates changed within the same clade.

The only discrepancies between trees inferred from ITS2 and 18S regions are in divergent positions of isolates 11 (*Macaca silenus* Liberec) and 16 (*Chlorocebus sabaeus* Liberec). These specimens are both classified as *T. suis* in the ITS2 tree (specifically in a novel subclade from primates; see Fig. 2), while 18S placed it within *T. trichiura* clade (Fig. 3).

Tree topology based on the *cox1* appears to be very unstable, probably because of the low information content of the partial sequence. Analysis of the *cox1* from species of *Trichuris* (Fig. 4) divided isolates from humans and non-human primates into two clades representing *T. trichiura* and *T. suis*, with the isolate from *Papio hamadryas* (isolate 7) on the basis of clades. However, the positions of more individual isolates, as revealed by *cox1* (Fig. 4), differed from those observed in ITS2 (Fig. 2), 18S rDNA (Fig. 3) and extended *cox1* analyses (Fig. 5) in three respects.

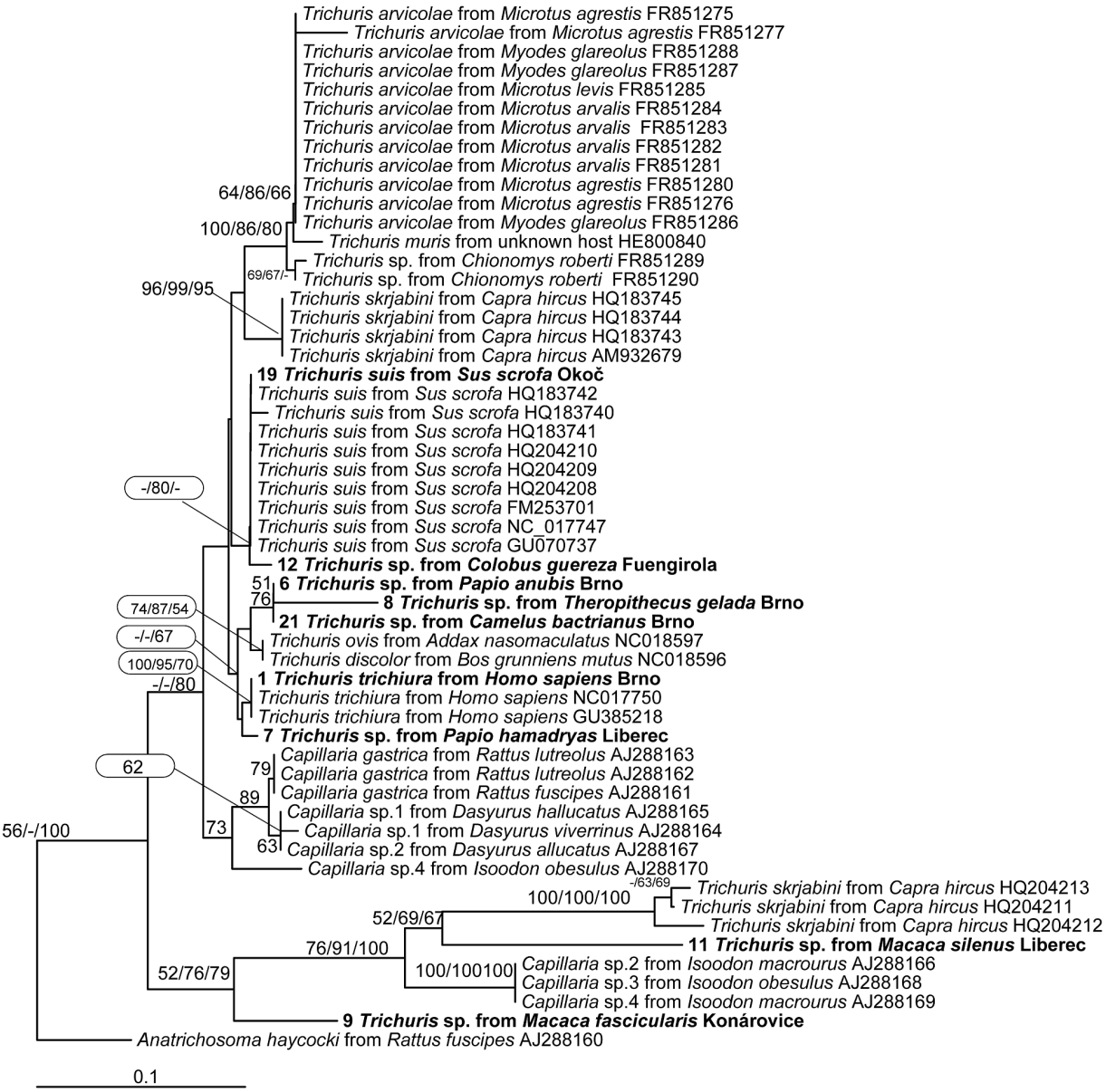

Fig. 5. Neighbor joining tree (by AsaturA, which is designed to deal with amino acid saturation) as inferred from *cox1* amino acid sequences using an extended data set. Numbers above branches indicate NJ bootstrap support (1 000 replicates)/ML bootstrap support (1 000 replicates)/MP bootstrap support (1 000 replicates). Sequences newly reported in this study are bold typed. Tree is rooted to *Anatrichosoma haycocki* Spratt, 1982 (AJ288160).

(1) The isolate from *Colobus guereza kikuyuensis* (isolate 12) appeared in two different positions according to the used method of tree reconstruction: (i) NJ analysis of the amino acid alignment (AsaturA) showed the unsupported affiliation of this isolate with *T. suis*; (ii) ML (PhyML) placed it in an unsupported position at the root of the *T. trichiura* cluster. Analysis based on the 18S rDNA (Fig. 3) and the combined dataset of all three genes confirmed the relationship of this isolate to *T. suis* (see Fig. S3); the ITS2 tree shows a very well supported affiliation of the *Colobus guereza* isolate to a diverse clade closely related to *T. suis* (Fig. 2).

(2) *Trichuris* from *Macaca fascicularis* (isolate 9) is in the ITS2 tree branching together with isolate from *Papio anubis* (isolate 6) within the *T. trichiura* clade. While 18S rDNA-based analysis placed these isolates as relative to *T. trichiura* as well, the *cox1* tree grouped isolate 6 (from *P. anubis*) with the *Theropithecus gelada* (isolate 8) and *Macaca silenus* (isolate 11) isolates in a substantially different position in close proximity to *T. skrjabini* (Fig. 4) and in extended *cox1* analyses to the genus *Capillaria* (Fig. 5). Thus, species of *Capillaria* appeared inside the genus *Trichuris*, making the genus *Trichuris* polyphyletic (Fig. 5).

(3) In the extended *cox1* tree (Fig. 5) within the *T. trichiura* clade, two subclades were formed: the first included isolates from humans and *Papio hamadrays* (isolate 7), whereas the second one was formed by isolates from *P. anubis* (isolates 6), *T. gelada* (isolate 8) and ruminants (*Trichuris ovis*, *T. discolor, Trichuris* sp. from camel). The ambiguity of *cox1* phylogeny of the genus *Trichuris* is underscored by the unexpected positions of *T. skrjabi-*

ni, which clustered in two substantially different clades (Fig. 5).

The concatenated tree constructed from partial sequences of the 18S, ITS2 and *cox1* sequences supported the division of the primate isolates into two clades (see Fig. S3). The position of whipworm isolates from *Macaca silenus* (11) and *C. guereza kikuyuensis* (12) within the *T. suis* clade and other primate isolates to the *T. trichiura* clade are congruent with the ITS2 analysis.

DISCUSSION

Similar to other nematodes, whipworm species are described and distinguished on the basis of morphological and biometrical features, the host(s) they infect and/or their geographical origin (Robles 2011, Torres et al. 2011). However, adherence to the morphological species concept requires the examination of well-preserved mature nematodes of both sexes, which are almost inaccessible from endangered mammals such as primates. Misleading classification could also obscure the zoonotic potential of whipworms.

Our study addresses to some extent the molecular diversity of isolates of *Trichuris* using partial sequences of nuclear and mitochondrial genomic sequences. The majority of phylogenetic studies on whipworms have been based on analyses of ITS1-5.8S-ITS2 rDNA, which revealed the relationships of rodent- and ruminant-infecting species of *Trichuris* (e.g. Cutillas et al. 2004, Callejón et al. 2012). However, the occurrence of more variants of RNA genes, including ITS2 region (Nissen et al. 2012), makes their use in solving the phylogeny of *Trichuris* much less informative, especially in organisms with unknown ploidy.

Nissen et al. (2012) detected two variants of ITS2 sequences in some individuals of *Trichuris* isolated from humans, one showing an affiliation with *T. suis* whereas the other clustering with *T. trichiura*. They proposed existence of two distinct genotypes in humans, which were probably due to cross-infections of man with *T. suis* and consequent hybridisation of these two species.

In contrast, Ravasi et al. (2012) suggested that these results were due to cross-contamination with genomic DNA of *T. suis*. Being aware of these issues, we have tested randomly selected isolates of *Trichuris* for the presence of gene variants by cloning and sequencing individual clones. Furthermore, the quality of the sequence reads from direct sequencing of PCR products was assessed (data not shown). We found no indication for more variants of the target genes or contamination.

Our phylogenetic analyses of whipworms from primates have shown the presence of two independent evolutionary lineages: (i) the *T. trichiura* clade and (ii) a clade primarily containing *T. suis*. This trend was also observed in isolates from humans, non-human primates and pigs (Liu et al. 2012, Nissen et al. 2012, Ravasi et al. 2012, Callejón et al. 2013). The majority of sequences (both from captive and free-ranging primates) within the *T. trichiura* clade can be classified as *T. trichiura* sensu stricto. Such an arrangement supports the zoonotic potential of *T. trichiura* and its ability to infect a broader range of primates (Munene et al. 1998, Phillips et al. 2004, Lee et al. 2010).

The *T. suis* clade, including whipworm sequences from pigs and primates, further branched into two subclades. The isolates from European, Asian and African domestic pigs and humans formed a highly geneticaly uniform group that could be unequivocally considered as *T. suis* sensu stricto. The isolates from one human and Old World primates of the second subclade might represent different taxon/taxa of *Trichuris*. Obviously, the isolates referred to as *T. trichiura* isolated from humans (Ravasi et al. 2012) and *Nomascus gabriellae* (see Cutillas et al. 2009) should be reclassified as was performed by isolate from *Colobus guereza kikuyuensis* (see Cutillas et al. 2014).

To overcome troubles with nuclear rDNA-based phylogenetic analyses, mitochondrial DNA sequences have become widely used (e.g. Blouin et al. 1998, Peng et al. 2007, Jex et al. 2009), allowing high resolution in deep analyses of closely related lineages (Callejón et al. 2012). However, when comparing rDNA and *cox1* data alone, support of branches is higher in trees based on nuclear data. Moreover, we obtained different topologies of some isolates: ITS2 placed an isolate from *Macaca silenus* (isolate 11) and *Chlorocebus sabaeus* (isolate 16) to the sister position to *T. suis*, whereas 18S rDNA analysis shows an affiliation of these isolates to *T. trichiura*. Phylogenetic position (NJ and ML tree) of the isolate from *M. silenus* (isolate 11) as inferred from *cox1* (Fig. 4) was among the isolates from ruminants and appeared in wider phylogenetic context in close proximity to the genus *Capillaria* (Fig. 5).

The presence of the genera *Capillaria* and *Anatrichosoma* in *cox1* analyses revealed an unexpected finding: isolates of *Capillaria* branched inside those of *Trichuris*, making thus this genus polyphyletic. Especially, *Trichuris* isolates from *M. silenus* (11) and *Macaca fascicularis* (9) formed a highly diverse clade together with three isolates of *T. skrjabini* from *Capra hircus* (HQ204211; HQ204212; HQ204213) and three sequences from *Capillaria* spp. The rest of sequences of *Capillaria* spp. branched at the root of the core taxa of *Trichuris*, which are defined here as species of *Trichuris* after exclusion of the diverse basal clade (see Fig. 5).

Similarly, *T. skrjabini* appears in two different positions in the *cox1* tree, three isolates were placed in the diverse basal cluster, while four others are in an advanced position as a sister group to *T. arvicolae*. Such a topology can be caused by the maternal heredity of mitochondrial genes (see Fig. S4 for details) or these isolates from a presumably diverse clade (HQ204211; HQ204212; HQ204213) were simply misclassified. However, since sequences HQ183745 and HQ204213 were obtained from a single isolate (GenBank information), maternal mitochondrial heredity and hybridisations might have affected the phylogeny of the genus. Although mitochondrial DNA is proposed to be used for phylogenetic and interspecific analyses in some nematodes (Liu et al. 2012, Jabbar et al. 2013), discrepancies in our analyses may argue against its use.

Although these discrepancies can also result from the low number of available species in both genera *Trichuris* and *Capillaria*, from the erroneous determination of species of *Capillaria* or from the presence of vestigial mitochondrial genes recently transferred to the nucleus (the so-called 'numts') (Richly and Leister 2004), we prefer a scenario involving hybridisation, ancient gene or genome duplication (Cardoso et al. 2006) or ancestral polymorphism (as explained in Fig. S5). It should be also noted that due to low sequence variability within mitochondrial *cox1* DNA, the use of a partial gene region (Liu et al. 2013) is likely not efficient to solve phylogenetic relationships among *Trichuris* spp. and related species. This notion is supported by the high variability of the topologies obtained by the three methods (see Figs. 4, 5). However, all used methods revealed the polyphyletic nature of *Trichuris*.

We combined nuclear and organellar DNA markers, which is recommended as a suitable phylogenetic approach, as the differences between the markers can enrich the interpretation of the evolutionary history of nematodes (e.g. Rubinoff and Holland 2005, Zarlenga et al. 2006). Our study represents the first attempt to join 18S, ITS2 and *cox1* markers in a concatenated analysis of *Trichuris*. However, the resultant dataset was drastically reduced due to the paucity of sequences in the GenBank Database. The isolates were divided into two groups roughly comprising human-primate and pig isolates, with two isolates from primates (*C. guereza kikuyuensis* and *M. silenus*) branching at the root of the pig clade (see Fig. S3).

So far, the whipworms referred as *T. trichiura* or *Trichuris* sp. were reported from 10 families of primates across three continents (e.g. Michaud et al. 2003, Phillips et al. 2004, Cutillas et al. 2009, Clough 2010, Rarivololona and Ganzhorn 2010). Considering the diversity of *Trichuris* spp. affecting other mammalian groups, such as rodents and ruminants (e.g. Cutillas et al. 2004, Robles 2011), it is unlikely that primates are infected with only one or two species of whipworms, as suggested in previous studies (Ravasi et al. 2012, Liu et al. 2013, Cutillas et al. 2014).

Our ITS2 analysis revealed three genetically different isolates of *Trichuris* from baboons scattered in both of the described major lineages. *Trichuris cynocephalus* was, until this year (Cutillas et al. 2014), the only species other than *T. trichiura* named from the primate suborder Haplorhini. This taxon was described based on subtle differences in whipworm morphology from captive yellow baboons *Papio cynocephalus* Linnaeus, by Khera (1951).

However, despite our morphological analyses of an isolate from *Papio hamandryas* (data not shown), we are not able to associate any particular sequences from baboons with *T. cynocephalus*. Thus, the question of the identity and validity of this species needs the study of more isolates from free-ranging *Papio* spp. Obviously, morphological analyses of adult whipworms are an essential complement to the molecular data as shown by Cutillas et al. (2014).

Evidence for more species of *Trichuris* in primates also has practical implications for epidemiology. A great deal of whipworms eggs found in the faeces of non-human primates have been classified as *T. trichiura* (e.g. Ooi et al. 1993, Reichard et al. 2008, Lee et al. 2010). Such a tentative placement may imply the broad zoonotic cross-transmission between non-human primates and humans (e.g. Munene et al. 1998, Chapman et al. 2006), which can be misleading in most cases. In contrast, limited experimental data have demonstrated the zoonotic transmission of *T. trichiura* between non-human primates and man (Horii and Usui 1985, Imada et al. 1986).

This finding is consistent with the close proximity of some of our isolates of *Trichuris* from primates and human-originated *T. trichura* (Fig. 2). However, most of the *Trichuris* isolates sequenced are from captive hosts and various *Trichuris* spp. might differ in their host specificity/preferences in free ranging populations (see Poulin and Keeney 2008). In the wild, various species of primates often overlap in their ranges and share habitats and/or foraging sites. Experimental transmission studies in primates to address host specificity of parasites are almost impossible, but careful selection of model field sites and a survey of the genetic diversity of species of *Trichuris* in such natural multi-primate communities can answer ongoing questions about host specificity.

Although named almost 250 year ago, whipworms of man and primates deserve more attention from epidemiologists. The obvious existence of more different species in man, broad distribution of trichuriasis in humans together with unresolved zoonotic aspects of 'new' species call for further research to address the diversity of whipworms in these hosts.

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