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Genetic heterogeneity and phylogeny of *Trichuris* spp. from captive non-human primates based on ribosomal DNA sequence data

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ABSTRACT

Nematodes of the genus *Trichuris*, known as whipworms, are recognized to infect numerous mammalian species including humans and non-human primates. Several *Trichuris* spp. have been described and species designation/identification is traditionally based on host-affiliation, although cross-infection and hybridization events may complicate species boundaries. The main aims of the present study were to genetically characterize adult *Trichuris* specimens from captive Japanese macaques (*Macaca fuscata*) and grivets (*Chlorocebus aethiops*), using the ribosomal DNA (ITS) as molecular marker and to investigate the phylogeny and the extent of genetic variation also by comparison with data on isolates from other humans, non-human primates and other hosts. The phylogenetic analysis of *Trichuris* sequences from *M. fuscata* and *C. aethiops* provided evidences of distinct clades and subclades thus advocating the existence of additional separated taxa. Neighbor Joining and Bayesian trees suggest that specimens from *M. fuscata* may be distinct from, but related to *Trichuris trichiura*, while a close relationship is suggested between the subclade formed by the specimens from *C. aethiops* and the subclade formed by *T. suis*.

The tendency to associate *Trichuris* sp. to host species can lead to misleading taxonomic interpretations (i.e. whipworms found in primates are identified as *T. trichiura*). The results here obtained confirm previous evidences suggesting the existence of *Trichuris* spp. other than *T. trichiura* infecting non-human living primates.

1. Introduction

Parasites represent a major threat for animals housed in zoos where stress conditions and high density in confined environments greatly facilitate their transmission, especially in case of direct life cycles (Naidu, 2000; Gracenea et al., 2002). Moreover, many of the parasites commonly found in zoological gardens are responsible for zoonoses, thus representing a potential public health problem (Klaus et al., 2000; Berrilli et al., 2011). Among these, nematodes of the genus *Trichuris* known as whipworms are recognized to infect several mammalian species, including humans (Reichard et al., 2008). Infection by *Trichuris* spp. is one of three major groups of soil-transmitted helminthiases (STHs) affecting 600 million people worldwide (Bethony et al., 2006) and, with the other STHs *Ascaris* spp. and hookworms, have a strong adverse effect on the socioeconomic development of populations. Trichuriasis is considered a Neglected Tropical Disease (NTD) by WHO, widespread in tropical and sub-tropical regions, especially in developing countries (Bethony et al., 2011; Hotez et al., 2012). The genus *Trichuris* includes around one hundred recognized species (Yamaguti, 1961), but only three, *Trichuris trichiura*, *Trichuris suis* and *Trichuris vulpis*, are considered zoonotic, representing a potential threat to human health (Ravasi et al., 2012). However, due to morphological and biological similarities, traditional specific identification based on morphology of both eggs and adult worms is not reliable and molecular tools would be highly recommended for this purpose. Whipworms are common parasites in captive non-human primates (NHP), where they are typically assumed to belong to the species *T. trichiura*. In fact, *Trichuris* species are considered to specifically infect a particular host species or a group of related hosts, and species designation/identification is mostly based on host affiliation. However, considering that cross-infection and hybridization events are quite frequent (Nissen

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et al., 2012), species boundary is not easy to define. In addition, recent molecular studies have described two distinct *Trichuris* genotypes infecting both humans and NHP (Ravasi et al., 2012; Liu et al., 2013), without clearly defining whether *Trichuris* sp. reported in captive and wild NHP are one or more different species. Additionally, *Trichuris colobae* has been recently described as new species from the colobus monkey *Colobus guereza kikuyensis*, being the first species to be formally designated from NHP (Cutillas et al., 2014).

In this complex and still uncertain taxonomic scenario, the main aims of the present study were: (i) to identify at species level adult specimens of *Trichuris* sp. from captive Japanese macaques (*Macaca fuscata*) and grivets (*Chlorocebus aethiops*) living at the Bioparco zoo of Rome, using the ribosomal DNA (ITS) as molecular marker; (ii) to investigate genetic variation in comparison to data on *Trichuris* spp. from non-human primates, humans and other hosts retrieved from GenBank.

2. Materials and methods

2.1. Collection of material

Trichuris sp. adult specimens were collected during necropsies carried out at the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" to ascertain the cause of death of two *M. fuscata* and one *C. aethiops*, formerly housed in the Bioparco of Rome, in strict accordance with good animal practices and veterinary inspection procedures.

Adult whipworms from each host were washed in physiological saline, morphologically identified as *Trichuris* sp. according to Jenkins (1970) and Ooi et al. (1993) and fixed in 70% ethanol until molecular analyses. Total genomic DNA was isolated from 19 specimens from *M. fuscata* and from 7 specimens from *C. aethiops* using the Wizard Genomic DNA Purification kit (Promega), according to instructions. The amplification of ITS1-5.8S-ITS2 region was performed as in Ravasi et al. (2012), including positive and negative control to each run. Positive amplicons were purified using Sure Clean (Bioline) and shipped to external service for sequencing (MWG Eurofins Operon).

2.2. Sequencing and phylogenetic analyses

A total of 26 nucleotide sequences of the entire ITS region of the nuclear ribosomal DNA were obtained (19 from two Japanese macaque *M. fuscata*, and 7 from one grivet *C. aethiops*). Data were compared to available sequences in GenBank of the genus *Trichuris* and aligned using web-PRANK (Löytynoja and Goldman, 2005), to generate four distinct datasets. Ambiguous sites/regions in the alignment were excluded using consistency alignment filter at 60%.

Dataset 1 included the 26 sequences from this study; Dataset 2 included also available sequences of *Trichuris* from humans and other primates. In Dataset 3, sequences from other host species rather than primates were also considered, while Dataset 4 was based on the ITS2 fragment using information from Dataset 3, plus additional data available for the ITS2. A representative sequence of *Ascaris lumbricoides* (Accession Number AB571298) was used as outgroup for all analyses. Information about specimens sequenced in the present study, accession numbers and GenBank retrieved sequences are available in Table 1. Sequences generated in the present research were deposited in GenBank, under the accession numbers KP336459-77 for specimens from *M. fuscata* and KP336478-84 for specimens from *C. aethiops*.

The software jModeltest (Posada, 2009) was used to compare the fit of nucleotide substitution models using the Akaike Information Criterion (AIC) under a total of 83 models, corresponding to 11 different schemes. The best-fit model and parameters determined for all datasets were then used for the Bayesian analyses. The Bayesian analyses were performed using the TN model (as selected by jModelTest), using BEAST software (Drummond and Rambaut, 2007); datasets were run twice for 10^6 generations, sampling the chain every 1000 generations. 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 10^2 generations. A Neighbor Joining analysis was performed for all datasets using MEGA6 (Tamura et al., 2013): the evolutionary distances were computed using the TN model and statistical reliability of nodes was evaluated using 1000 pseudoreplications bootstrap.

Using phylogenetic clade affiliation as a criterion to test the hypothesis of their existence, specimens were grouped according to clades previously reported (Ravasi et al., 2012; Callejón et al., 2013) and to those observed strictly in the present study, as indicated in Table 1: four groups corresponding to subclades CA, suis, DG and MF were created to estimate the fixation indices F_{st}, using Arlequin (Excoffier et al., 2007) and to evaluate the evolutionary divergence over sequence pairs between groups using MEGA6.

3. Results

Phylogenetic analyses and evaluation of evolutionary distances between taxa revealed the existence of new taxonomic entities and confirmed the existence of previously identified clades.

Phylogenetic trees obtained from the NJ and the Bayesian approaches gave the same topology for all Datasets analyzed, except for a slight deviation reported in a branch of Dataset 3 trees.

Trees obtained from the Dataset 1 evidenced a clear-cut separation into two clades, one comprising *Trichuris* spp. from *M. fuscata* and one including those from *C. aethiops*, supported both by 100% bootstrap and posterior probability values.

The phylogenetic analyses of the Dataset 2 confirmed the separation of the two former clades. In particular, specimens from *M. fuscata* (designated as subclade MF) showed a close relationship with retrieved GenBank sequences corresponding to clade *Trichuris* sp. DG of Ravasi et al. (2012), probably to Clade 2 of Callejón et al. (2013) and to Group 1 of Ghai et al. (2014), which contains *T. trichiura* from humans from China and from *Papio ursinus* from South Africa.

Specimens from *C. aethiops* and two sequences of *T. trichiura*, one from a human case from Cameroon and one from *P. ursinus* from South Africa, formed a separated group renamed subclade CA, probably corresponding to Clade 1 of Callejón et al. (2013), as better evidenced in results from the Dataset 3.

Two retrieved sequences of *T. trichiura* from *Nomascus gabriellae* and from *C. guereza kikuyensis* were found to be sister taxa of the *C. aethiops* subclade. The same topology was also described by Ravasi et al. (2012), who defined this group as *Trichuris* sp. CP-GOB, including also specimens of *T. suis*.

Nodes separating the above subclades and clades were supported by high bootstrap/posterior probability values.

The analysis of the Dataset 3 including sequences from other non-primates mammalian hosts revealed slightly different topologies inside the branching of Clade 1 – CP-GOB (Fig. 1). Both NJ and Bayesian trees depicted a close relationship between the subclade formed by the specimens from *C. aethiops* (subclade CA) and the subclade formed by *T. suis* (subclade suis included into the Clade 1 in Callejón et al., 2013). However they seem to be significantly separated, suggesting two distinct evolutive lineages. While the group including *Trichuris* sp. from humans (subclade DG in Clade 2) and from *M. fuscata* (subclade MF in Clade 2) remained unaltered as in the Dataset 2, alternative position in Clade

Table 1
Data on specimens analyzed in the present paper at nuclear ribosomal DNA (ITS) are reported, together with sequences retrieved from GenBank used for comparison. GenBank accession numbers, *Trichuris* species, host species and phylogenetic clade affiliation are reported in columns. Accession numbers of sequences generated in the present paper are in italics. The symbol " indicates that information is as above in the same column.

GenBank Accession number	*Trichuris* putative species	Host species	Phylogenetic Clade
KP336459-77	*Trichuris sp.*	*Macaca fuscata*	Subclade MF
AB586133	"	"	"
KP336478-84	*Trichuris sp.*	*Chlorocebus aethiops*	Subclade CA
JF690949-50	"	"	"
GQ301551-53	*Trichuris sp.*	*Papio ursinus*	Subclade DG
GQ301554	*Trichuris trichiura*	"	Clade1-CP GOB
KJ588152, KJ588154-55	*Trichuris trichiura*	*Papio anubis*	Subclade CA
KJ588163-64	*Trichuris trichiura*	*Cercopithecus lhoesti*	Subclade CA
AM992981, AM992984	*Trichuris trichiura*	*Homo sapiens*	Subclade DG
AM992987, AM992990	"	"	"
AM992993, AM992996	"	"	"
KJ588133	*Trichuris sp.*	"	"
KJ588132	*Trichuris trichiura*	"	"
JN181860	"	"	"
JF690940	"	"	"
GQ301555	"	"	Subclade CA
KJ588151	"	"	"
KJ588159	"	"	"
JN181822	"	"	Subclade suis
JF690946	*Trichuris sp.*	*Macaca fascicularis*	Subclade DG
KJ588135	*Trichuris trichiura*	*Cercopithecus ascanius*	Subclade CA
KJ588145	"	"	"
KJ588148	"	"	"
KJ588150	"	"	"
KJ588153	"	"	"
KJ588138	*Trichuris trichiura*	*Cercopithecus mitis*	Subclade CA
KJ588141	"	"	"
KJ588143	"	"	"
KJ588156	"	"	"
KJ588165	"	"	"
FM991955	*Trichuris trichiura*	*Nomascus gabriellae*	Clade1-CP GOB
FM991956	*Trichuris colobae*	*Colobus guereza*	Subclade CA
KJ588136	"	"	"
KJ588139	"	"	"
KJ588146	"	"	"
KJ588149	"	"	"
KJ588157-58	"	"	"
KJ588161	"	"	"
KJ588166-67	"	"	"
KJ588137	*Trichuris trichiura*	*Procolobus rufomitratus*	Subclade CA
KJ588140	"	"	"
KJ588142	"	"	"
KJ588144	"	"	"
KJ588162	"	"	"
KJ588147	*Trichuris trichiura*	*Lophocebus albigena*	Subclade CA
KJ588160	*Trichuris trichiura*	*Pan troglodytes*	Subclade CA
AM992999-3000	*Trichuris suis*	*Sus scrofa*	Subclade suis
AM993003-8	"	"	"
AM993011-12	"	"	"
AM993014-16	"	"	"
AJ249966	"	"	"
AJ489248	*Trichuris skrjabini*	*Capra hircus*	Clade 3
AB367794	*Trichuris discolor*	*Capricornis crispus*	"
HE608854	*Trichuris discolor*	*Bos taurus*	"
FR870274	*Trichuris ovis*	*Bos taurus*	"
JF680987	*Trichuris ovis*	*Ovis aries*	"
AM234616	*Trichuris vulpis*	*Canis lupus familiaris*	Clade 4
FN543185	*Trichuris arvicolae*	*Myodes glareolus*	"
FN543200	*Trichuris muris*	*Mus domesticus*	"
EU276016	*Trichuris muris*	*Apodemus sylvaticus*	"
AB571298	*Ascaris lumbricoides*	*Homo sapiens*	Outgroup

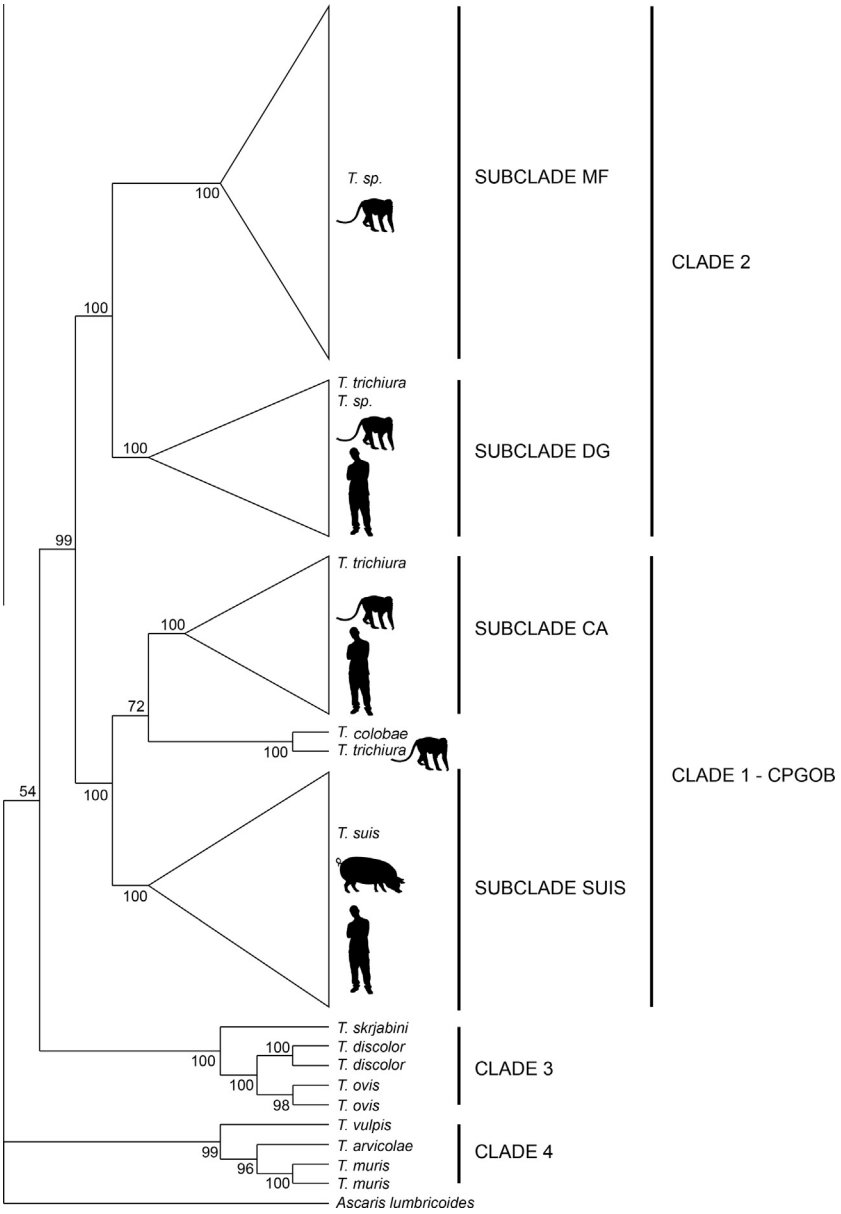

Fig. 1. Bayesian tree based on the analysis of the Dataset 3 including sequences of the entire ITS from other non-primates mammalian hosts, with indications on host affiliation and assignment to clades and subclades, following the nomenclature used in previous papers (Ravasi et al., 2012; Callejón et al., 2013).

1 – CP-GOB of the two sequences from *N. gabriellae* and from *C. guereza kikuyensis* is observed: in the NJ tree, they are the sister group of specimens from *C. aethiops* (subclade CA) and *T. trichiura* from human and *P. ursinus* (GQ301554-55), excluding *T. suis* subclade but in the Bayes tree they are the sister group of the entire Clade 1. The topology relative to Clade 2 revealed a clear distinctiveness between the individuals from *M. fuscata* (subclade MF) and the sequences referred as to *T. trichiura* from humans and other primates, suggesting the existence of two distinct taxa.

Two additional clades were detected, one comprising *Trichuris* spp. from herbivores (Clade 3) and one from rodents and dogs (Clade 4).

The analysis of the Dataset 4, based on the only ITS2 region, gave identical topologies using the NJ and Bayesian approaches with a picture of phylogenetic relationships largely overlapping the results obtained from the entire ITS, although permitting a more comprehensive comparison with samples from other NHP (Fig. 2). Solely the sequences from *M. fuscata* available in GenBank for the same genetic region is in fact included in the subclade MF together with all sequences from *M. fuscata* here analyzed.

Results obtained from pairwise comparisons of taxonomic groups defined using subclade affiliation as criterion (subclades MF, DG, CA and suis), revealed high level of differentiation with F_{st} values ranking from 0.78 to 0.90, as expected for well separated species. Similarly, *p*-distance estimations over sequences pairs showed evolutionary divergence values from 0.05 to 0.38. All F_{st} values obtained were statistically significant (significance level: 0.05).

4. Discussion

The use of molecular markers is particularly useful in the identification of taxa when morphology is not sufficient to discriminate among very closely related species, especially when most variation in morphological features is associated with apparent adaptation (such as recent adaptive radiations) or when species separation is too recent. In addition, morphological convergence is a very

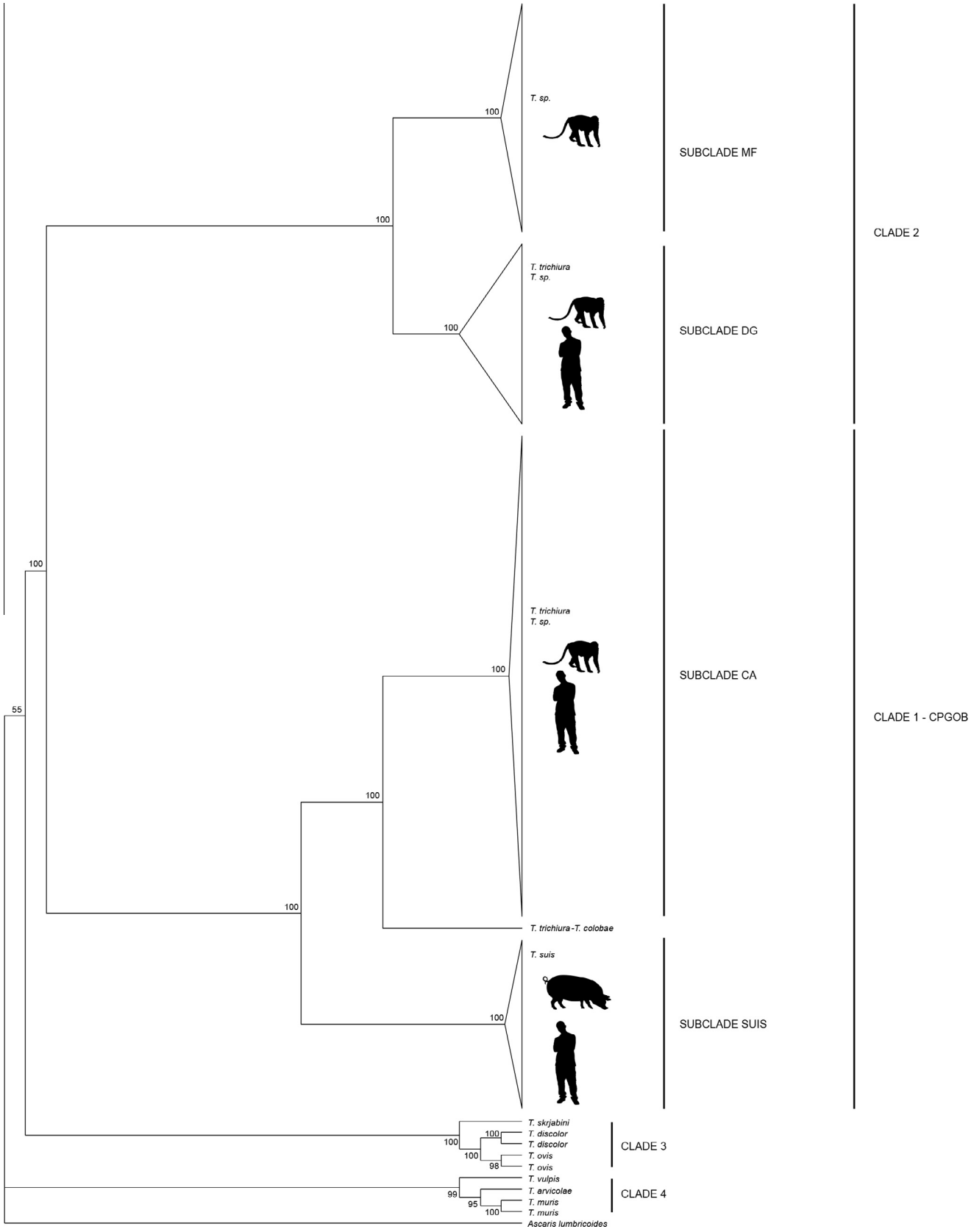

Fig. 2. Bayesian tree based on the analysis of the Dataset 4 including sequences of the ITS-2, with indications on host affiliation and assignment to clades and subclades.

common phenomenon in intestinal parasitic nematodes (i.e. *Anisakis* spp. and *Ascaris* spp.) and also the genus *Trichuris* shows limitations in availability of morphological diagnostic characters.

Nuclear ribosomal DNA coding and non-coding regions have been widely used for molecular systematics and phylogeny in parasitic nematodes showing higher inter-specific than intra-specific

variability, allowing the description of new species, the discovery of cryptic/sibling species and of putative hybrids between very closely related species, e.g. in ascaridoid nematodes (Abollo et al., 2003; Peng et al., 2003; Cavallero et al., 2014). Cross-infection and hybridization between human and porcine species have been reported also in *Trichuris* spp. (Nissen et al., 2012).

In the present study, analyses of *Trichuris* sp. ribosomal sequences from *M. fuscata* and *C. aethiops* for phylogenetic and evolutionary distance estimations suggested their existence as additional separated taxa. Values obtained from F_{st} and *p*-distance support this hypothesis, revealing well-differentiated groups. These results are in agreement with previous evidences obtained using the same molecular markers as well as other nuclear and mitochondrial regions where most of *Trichuris* spp. seem to show multi-host affiliation that underlie the taxonomic separation (Callejón et al., 2010; Liu et al., 2012). In fact, as previously suggested, *Trichuris* spp. may exist as a complex of species with different host affinity and cross-infection capability. Host affiliation should not be considered as a feature of definitive systematic value, since each subclade includes individuals from more than one host-species, except for the subclade MF including only specimens from macaques.

Limitations on unambiguous species identification rise from the tendency to associate *Trichuris* sp. with host species: whipworms found in primates are routinely, and often uncritically, identified as *T. trichiura*. The results here obtained confirm previous evidences suggesting the existence of *Trichuris* sp., other than *T. trichiura* infecting non-human living primates. More specifically, *Trichuris* sp. from *C. aethiops* are more closely related to *T. suis* rather than to other *Trichuris* from primates. Also, the two sequences retrieved from GenBank defined as *T. trichiura* from *Colobus* and *Nomascus* with uncertain position in relation to Clade 1 – CP-GOB may be considered as separated in view of values of pairwise estimations and of recent outcomes that led to defining the specific status of *T. colobae* (Cutillas et al., 2014).

The comparison of *Trichuris* spp. phylogeny with a recent study on comparative genomics of living primates (Perelman et al., 2011) suggests a possible concordance between host affiliation of *Trichuris* sp. and phylogenetic relationship of primates, even at a different taxonomic scale (genus-family). However, the host affiliation observed is not strictly specific, since human and baboon are included in two clades and may be infected by more than one *Trichuris* species.

The Clade subdivision based on host nutritional requirements suggested by Callejón et al. (2012) does not completely explain independent clustering into two phylogenetic lineages of *Trichuris* sp. from human and non-human primates and of *T. suis*, but it probably reflects ancient association between human and pigs as a consequence of domestication.

With the advent of Next Generation Sequencing technologies additional genetic data will be soon available for genomic comparisons at intra-specific and population level (Foth et al., 2014; Ghedin, 2014), allowing to increase resolution power to address important biological issues, including the identification of further markers potentially useful for the discrimination of very closely related species. To clarify this point is even more important considering the therapeutic use of *T. suis* eggs for auto-immune disease treatment (Berrilli et al., 2012; Jouvin and Kinet, 2012), and the recovery of adult worms in a patient treated with eggs (Kradin et al., 2006). *T. suis* eggs are morphologically very similar to *T. trichiura* eggs and cases of cross-infection may be undetected by standard methods (Nejsum et al., 2012). Moreover, elucidating the taxonomic relationship between the closely related human and baboon *Trichuris* spp. may be important for using baboon as a model for human trichuriasis, as suggested by Hansen et al. (2013).

The recovery of parasites in zoological gardens raises questions regarding: (i) the route of parasites introduction in an isolated structure/group of hosts; (ii) the management measures to prevent cyst/eggs transport from one enclosure to another within the zoological garden; (iii) the zoonosic risk.

As for the case here described of *Trichuris* sp. infecting *M. fuscata* and *C. aethiops* in the Bioparco, the use of molecular tools to identify whipworms at species level have helped to address these gaps. In fact, an infection with murine, human or pig whipworms with rats or via contaminated food could commonly explain the parasite's arrival route into the zoological garden. Results of molecular characterization allowed to exclude these hypotheses at least for *Trichuris* sp. found in *M. fuscata*, indicating that these primates were presumably infected by their specific wild whipworms before captivity.

5. Conclusions

Genetic heterogeneity and phylogeny within the genus *Trichuris* from captive Japanese macaque and grivet provide evidences for the existence of distinct clades following analysis of nuclear ribosomal marker (ITS). The obtained results confirmed the existence of *Trichuris* spp. other than *T. trichiura* infecting non-human living primates. The correct species assignment and identification represents an important basis to the definition of host affiliation, the detection of transmission routes and for the establishment of management plans and control measures in captive primates.

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