DOI 10.2478/s11687-014-0214-y

HELMINTHOLOGIA, 51, 2: 83 – 93, 2014

Prevalence and genetic diversity of *Oesophagostomum stephanostomum* in wild lowland gorillas at Moukalaba-Doudou National Park, Gabon

P. MAKOULOUTOU[1], P. P. MBEHANG NGUEMA[2], S. FUJITA[3], Y. TAKENOSHITA[4], H. HASEGAWA[5], T. YANAGIDA[1], H. SATO[1,*]

[1]Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan, [*]E-mail: *sato7dp4@yamaguchi-u.ac.jp*; [2]L'Institut de Recherches en Ecologie Tropicale (IRET), Le Centre National de la Recherche Scientifique et Technologique (CENAREST), Libreville, Gabon; [3]Department of Basic Veterinary Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan; [4]Faculty of Child Studies, Chubu Gakuin University, Gifu, Japan; [5]Department of Biology, Faculty of Medicine, Oita University, Oita, Japan

Summary

Using a sedimentation method, the prevalence of the nodular worm *Oesophagostomum stephanostomum* (Nematoda: Strongylida) in western lowland gorillas at Moukalaba-Doudou National Park (MDNP), Gabon, was determined in fecal samples collected between January 2007 and October 2011, along with their coprocultures. Concurrently, possible zoonotic *Oesophagostomum* infections in villagers living near MDNP were assessed from their fecal samples collected during October and November of 2011. In the gorillas, strongylid (*Oesophagostomum* and/or hookworm) eggs were found in 47 of 235 fecal samples (20.0 %) and *Oesophagostomum* larvae were detected in 101 of 229 coprocultures (44.1 %). In the villagers, strongylid eggs were found in 9 of 71 fecal samples (12.7 %), but no *Oesophagostomum* larvae were detected in coprocultures. The internal transcribed spacer (ITS) region of ribosomal RNA gene (rDNA) and cytochrome *c* oxidase subunit-1 (*cox-1*) region of mitochondrial DNA (mtDNA) of coprocultured *Oesophagostomum* larvae were amplified using parasite DNA extracted from 7 – 25 larvae/sample, cloned into *Escherichia coli*, and sequenced. Sequenced rDNA contained 353/354-bp long ITS1, 151-bp long 5.8S rDNA, and 227-bp long ITS2. Parts of clones showed variations at 1 – 3 bases in the ITS1 region at a frequency of 24/68 (35.3 %) and at 1 – 2 bases in the ITS2 region at a frequency of 7/68 (10.3 %), whereas the 5.8S rDNA was essentially identical. Sequenced *cox-1* gene of the parasites, 849 bp in length, showed a higher number of nucleotide variations, mainly at the third nucleotide position of the codon. The majority of clones (27/41 (65.9 %)) had an identical amino acid sequence. These results suggest that at MDNP, Gabon, only a single population of *O. stephanostomum* with a degree of genetic diversity is prevalent in western lowland gorillas, without zoonotic complication in local inhabitants. The possible genetic variations in the ITS region of rDNA and *cox-1*gene of mtDNA presented here may be valuable when only a limited amount of material is available for the molecular species diagnosis of *O. stephanostomum*.

Keywords: *Oesophagostomum stephanostomum*; western lowland gorilla; Gabon; genetic diversity; internal transcribed spacer (ITS); *cox-1*

Introduction

Nematodes of the genus *Oesophagostomum*, termed nodular worms, cause oesophagostomiasis characterized by granuloma formation, caseous lesions or abscesses around encapsulated larvae in the intestinal wall of suids, ruminants, primates, and African rodents (Chabaud & Durette-Desset, 1974; Lichtenfels, 1980; Stewart & Gasbarre, 1989; Anderson, 1992; Polderman & Blotkamp, 1995; Krief *et al.*, 2008). Clinical oesophagostomiasis is evident worldwide in domestic animals and focally or sporadically in humans (Stewart & Gasbarre, 1989; Polderman & Blotkamp, 1995). Of the eight *Oesophagostomum* spp. recorded to date from non-human primates, *O. bifurcum*, *O. stephanostomum*, and *O. aculeatum* have also been reported in humans (Chabaud & Lariviere, 1958; Polderman & Blotkamp, 1995). Zoonosis caused by *O. bifurcum* is endemic in the northernmost part of Ghana and Togo situated in western Africa, and high prevalences of the disease in human residents have been recorded (Blotkamp *et al.*, 1993; Polderman & Blotkamp, 1995; Pit *et al.*, 1999; Yelifari *et al.*, 2005; Gasser *et al.*, 2006). Recently, however, multifaceted genetic analyses have demonstrated that different genotypes of *O. bifurcum* are prevalent in human patients and local non-human primates (Mona monkeys, Patas monkeys, Green monkeys, and Olive baboons) in Ghana and Togo (de Gruijter *et al.*, 2004, 2005; reviewed by Gasser *et al.*, 2006, 2009), supporting parallel assump-

tions based on epidemiological and morphological analyses (van Lieshout *et al.*, 2005; de Gruijter *et al.*, 2006).
Genetic characterization of *Oesophagostomum* spp. facilitates not only reliable diagnosis of the species without morphological observation of the adult parasite, but also transmission dynamics of the parasite in different animal hosts distributed in the same area, as seen in preceding intensive studies on *O. bifurcum* (de Gruijter *et al.*, 2004, 2005; Gasser *et al.*, 2006, 2009). In the present study, we collected fecal samples from western lowland gorillas (*Gorilla gorilla gorilla*) and human residents at the Moukalaba-Doudou National Park (MDNP), Gabon, to evaluate the current status of oesophagostomiasis in local primates, i.e. gorillas and humans. Furthermore, *Oesophagostomum* isolates were genetically characterized based on the internal transcribed spacer (ITS) region of ribosomal RNA gene (rDNA) as well as the partial sequence of cytochrome *c* oxidase subunit-1 (*cox-1*) gene of mitochondrial DNA (mtDNA) in order to define the genetic diversity of locally prevalent *Oesophagostomum* parasites. Elucidation of the possible genetic variation of the most frequently used markers, namely the ITS region of rDNA and *cox-1* gene of mtDNA, should facilitate the specific diagnosis of parasites, particularly when limited numbers of specimens are available for this purpose.

Materials and methods

Study site and sample collection
Sample collection was carried out in a national park in southern Gabon, Moukalaba-Doudou National Park (2˚26'S, 10˚25'E), covering an area of 5,028 km^2 with tropical rain forest and savannah grasslands. It is home to western lowland gorillas and chimpanzees (*Pan troglodytes*), and a program for the habituation of a group of gorillas (Group Gentil, GG) localized mainly on the southwestern side of the park (approximately 30 km^2) has been successfully conducted since 2003 by researchers from Kyoto University, Japan, and their Gabonese colleagues of L'Institut de Recherches en Ecologie Tropicale (IRET) (Ando *et al.*, 2008). During the habituation and all-day follows of GG gorillas, fresh feces of the members, ca. 20 in total, were collected between January 2007 and October 2011 for this study, along with samples of gorillas belonging to unknown groups or solitary individuals and a few chimpanzees. Fecal samples of villagers living near the park were collected during October and November of 2011 with the permission of Le Centre National de la Recherche Scientifique et Technologique (CENAREST) and the chiefs of two villages. We explained the purpose of our study to all participants and obtained signed documents from every participant which allowed us to use their feces for research purposes. The Moukalaba River is a natural barrier between the villages and the home range of animals in the park, particularly great apes.
Individual fresh fecal samples of gorillas and villagers were divided into three parts: 1) one part was fixed in 10 % neutral-buffered formalin; 2) one part was fixed in 80 % ethanol; and 3) one part was used for coproculture as described below. Each sample tube was carefully labeled with collection date, host species, and location. Although individual identification of GG gorillas had been established (Ando *et al.*, 2008), individual ID numbers were not available for samples collected for this study.
A modified Harada-Mori fecal culture technique using a filter paper strip and a disposable tea bag according to Hasegawa (2009a) was conducted at an open research cabin in the park. After keeping the cocultures for 1 – 2 weeks, grown larvae that emerged into the water were fixed in 80 % ethanol.

Parasitological examination
Fixed fecal samples and coproculture products were transported to the Laboratory of Parasitology, Yamaguchi University, Japan, and microscopic examinations were performed as follows. An approximate 1 – 2 g amount of formalin-fixed feces was examined microscopically following processing with a standard egg sedimentation method. Referring to Hasegawa (2009b), helminth eggs were identified under a light microscope. Apart from trophozoites of the ciliate *Troglodytella abrassarti*, protozoan infections were not assessed in this study. Coprocultured nematode larvae were separated under a dissection microscope and detailed morphology was observed under a light microscope with reference to van Wyk *et al*. (2004).

DNA extraction, polymerase chain reaction (PCR), and sequencing
From individual fecal cultures, 7 – 25 *Oesophagostomum* larvae were arbitrarily selected and washed three times in distilled water. Parasite DNA was extracted from such a pool of larvae using an Illustra™ tissue & cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions.
PCR amplification of the entire ITS1 – 5.8S – ITS2 region of rDNA was performed using a combination of a forward primer (NC5/F2; 5'-GTAGGTGAACCTGCGGAAGGATCAT-3') and a reverse primer (NC2; 5'-TTAGTTTCTTTTCCTCCGCT-3') flanking the 3'-terminus of 18S rDNA and the 5'-terminus of 28S rDNA, respectively (Newton *et al.*, 1998). The following PCR cycling protocol was used: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 90 sec, followed by a final extension at 72˚C for 7 min, as performed previously (Makouloutou *et al.*, 2013). PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan), cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.) and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. For the majority of larval samples, at least three clones per culture were sequenced.

The partial *cox-1* gene of parasite mtDNA was amplified by a primer pair of StrCoxAfrF (5'-GTGGTTTTGGTAAT TGAATGGTT-3') and MH28R (5'-CTAACTACATAAT AAGTATCATG-3') described by Hasegawa *et al.* (2010), but a different cycling protocol was used as follows: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 51˚C for 45 sec, and 72˚C for 1 min, followed by a final extension at 72˚C for 7 min. Amplicons were sequenced after genomic cloning as detailed above.

In order to identify the species of filariform larvae in coprocultures, two overlapping fragments of 18S rDNA were amplified by primer pairs of S.r.18S-F1/S.r.18S-R1 and SSU22F/SSU23R and sequenced as described previously (Sato *et al.*, 2006). Parasite DNA was extracted from a pool of filariform larvae (8 – 50 filariform larvae, average 16.3) collected from individual coprocultures.

The newly obtained sequences of *Oesophagostomum* larvae and those of related strongylid species retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson *et al.*, 1994). Likewise, the newly obtained sequences of the filariform larvae and those of *Strongyloides* spp. retrieved from the aforementioned databases were similarly aligned. The nucleotide sequences reported in the present study are available in the DDBJ/EMBL/GenBank databases under accession numbers AB821013 – AB821046.

Putative secondary structure of ITS2

Based on the structural association between 5.8S and 28S rDNA (Hwang & Kim, 2000; Gottschling & Plötner, 2004), a putative secondary structure of the 5.8S rDNA of *Oesophagostomum* spp. was drawn. For predicting the secondary structure of ITS2 molecules using the energy minimization approach for each sequence, the mfold web server (http://mfold.rna.albany.edu/) (Zuker, 2003) and the University of Vienna's RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Hofacker, 2003; Gruber *et al.*, 2008) were employed.

cox-1 haplotype analysis

Relationships of different haplotypes based on *cox-1* nucleotide sequences were visualized using an automated haplotype network layout and visualization software, HapStar, downloaded at http://fo.am/hapstar (Teacher & Griffiths, 2011).

Results

Prevalence of gastrointestinal parasites in gorillas and human residents at MDNP

From microscopic examination of 205 fecal samples from GG gorillas and 30 fecal samples from gorillas belonging to other groups or solitary individuals, strongylid eggs were found at the highest frequency (average 20.0 %) among helminths (Table 1). Since fecal samples examined in this study could not be traced back to individual animals, and GG members were approximately 20 in total number, these prevalence data represent repeated examinations – on average 10 times – at different opportunities. In 71 villagers living near MDNP, whipworm infection was the most common helminthiasis, being prevalent in all four age and sex categories of people (23.1 – 47.4 %; Table 1). Strongylid eggs were also detected in the fecal samples of villagers, but mainly in men over 40 years of age (29.2 %).

In order to identify the strongylid species, coproculture of 229 fecal samples from gorillas and 19 fecal samples from villagers was performed (Table 2). Representing the highest frequency (44.1 %), *Oesophagostomum* larvae were detected in 101 coprocultures from gorillas, whereas hookworm larvae were found in 52 coprocultures (22.7 %). Filariform *Strongyloides* larvae were found in 35 coprocultures from gorillas (15.3 %). Partial 18S rDNA fragments, 1,142 bp in length, were successfully sequenced for 14 coprocultures of the feces obtained from gorillas, in addition to two coprocultures of the feces of chimpanzees.

Table 1. Microscopic examination of fecal samples from gorillas and human residents at MDNP, Gabon*

Host	**Western lowland gorillas (n = 235)**		**Villagers living near MDNP (n = 71)**			
	Habituated Group Gentil (GG)	**Other groups or solitary**	**Men, >40-yr-old**	**Women, >40-yr-old**	**Youths, 16 to 30-yr-old**	**Children, <15-yr-old**
No. of samples examined	205	30	24	19	15	13
Strongylid eggs	37 (18.0 %)	10 (33.3 %)	7 (29.2 %)	1 (5.3 %)	0	1 (7.7 %)
***Strongyloides* eggs**	13 (6.3 %)	0	0	0	0	0
***Ascaris* eggs**	1 (0.5 %)	0	1 (4.2 %)	3 (15.8 %)	2 (13.3 %)	1 (7.7 %)
***Trichuris* eggs**	1 (0.5 %)	1 (3.3 %)	10 (41.7 %)	9 (47.4 %)	6 (40.0 %)	3 (23.1 %)
***Fasciola*-like trematode eggs**	12 (5.9 %)	0	1 (4.2 %)	0	0	0
***Streptopharagus* eggs**	1 (0.5 %)	0	0	0	0	0
***Schistosoma haematobium* eggs**	0	0	0	1 (5.3 %)	0	0
Troglodytella abrassarti	86 (42.0 %)	9 (30.0 %)	0	0	0	0

*Number of positive samples (%)

Table 2. Coproculture of fecal samples from gorillas and human residents at MDNP, Gabon*

Host	Western lowland gorillas (n = 229)		Villagers living near MDNP (n = 19)			
	Habituated Group Gentil (GG)	Other groups or solitary	Men, >40-yr-old	Women, > 40-yr-old	Youths, 16 to 30-yr-old	Children, <15-yr-old
No. of samples examined	198	31	11	1	5	2
***Oesophagostomum* larvae**	88 (44.4 %)	13 (41.9 %)	0	0	0	0
Hookworm larvae	46 (23.2 %)	6 (19.4 %)	7 (63.6 %)	0	2 (40.0 %)	2 (100 %)
***Strongyloides* larvae**	33 (16.7 %)	2 (6.5 %)	3 (27.3 %)	0	1 (20.0 %)	0

*Number of positive samples (%)

All these 16 sequences were absolutely identical to each other, and to a deposited 18S rDNA sequence of *Strongyloides fuelleborni* from a Japanese mammalogist visiting Tanzania (DDBJ/EMBL/GenBank accession no. AB453320; Hasegawa *et al.*, 2010). Identities of the newly obtained sequence (accession nos. AB821045 and AB821046) with those previously recorded from a gorilla and a chimpanzee at MDNP (accession nos. AB453322 and AB453321, respectively) were 99.64 % and 99.38 %, respectively. From coprocultures of 19 villagers, only hookworm larvae and *Strongyloides* larvae were detected at rates of 57.9 % (11/19) and 21.1 % (4/19), respectively. Species identification of these nematodes was not performed in the present study.

Sheathed *Oesophagostomum* larvae obtained from coprocultures of gorillas' feces had characteristic morphological features: triangular intestinal cells, a very long and finely tapered sheath tail, and prominent transverse striations on the sheath throughout most of its length. These characters were used to differentiate them from hookworm larvae. Larvae (n = 7) excluding the sheath were 597 – 927 (average 689) µm long by 20 – 24 (22) µm wide. Larvae including the sheath were 791 – 977 (879) µm long by 28 – 32 (30) µm wide. The buccal cavity was short, 8.5 µm in depth; the esophagus was strongyliform, 136 – 152 (143) µm long; the nerve ring was located 88 – 113 (103) µm from the anterior end; the excretory pore was found 80 – 102 (91) µm from the anterior end; triangular intestinal cells were 30 to 32 in number; tails were almost conical, 61 – 72 (66) µm long; and the tail part of sheaths was 202 – 347 (242) µm long.

ITS1–5.8S–ITS2 rDNA sequence of Oesophagostomum larvae

An 827/828-bp long fragment was successfully amplified in 29 of 56 larval samples containing 4 – 34 (average 13.4) larvae/coproculture. Using these amplicons, 68 clones for 21 larval samples were established and sequenced; 3 – 6 clones for 12 samples, 2 clones for 6 samples, and one clone for 3 samples. Concurrently, 3 clones from the coproculture of a chimpanzee's fecal sample (6 larvae) were obtained.

Amplicons contained 353/354-bp long ITS1, 151-bp long 5.8S rDNA, and 227-bp long ITS2 sequences, based on putative secondary structures of each region of rDNA (not shown). The majority of clones showed an identical nucleotide sequence regardless of their origins (Table 3): 42/68 (61.8 %) for ITS1, 61/67 (91.0 %) for ITS2, and almost all (95.6 %) for 5.8S rDNA. In comparison to retrieved ITS2 sequences of nine *Oesophagostomum* spp., the sequences we obtained in the present study were almost identical to that of *O. stephanostomum* recovered from a chimpanzee in Tanzania (DDBJ/EMBL/GenBank accession no. AF136576), except for three polymorphic nucleotide positions 122, 182, and 203 in the deposited sequence (Table 3). Closely related ITS sequences were found in primate nodular worms, such as *O. bifurcum* (AF136575, Y11733) and *O.* cf. *aculeatum* (AB586134), and a nodular worm of sheep and goats, *O. columbianum* (AJ006150), but the identities of ITS2 sequences between these three species and the present isolates were less than 95.0 %. Furthermore, the ITS2 sequences of two other *Oesophagostomum* spp. of sheep and goats (*O. asperum* and *O. venulosum*) were evidently different, as were species of pigs (*O. dentatum* and *O. quadrispinulatum*) and cattle and water buffaloes (*O. radiatum*). As far as examined at present, the ITS1 sequences of *Oesophagostomum* spp. showed lower interspecific variations than the ITS2 region (Table 3).

cox-1 mtDNA sequence of Oesophagostomum larvae

An 895-bp long fragment containing 46-bp long primer regions was successfully amplified in 16 of 56 larval samples containing 6 – 25 (average 12.5) larvae/coproculture. Using these amplicons, 36 clones for 12 larval samples were established and sequenced; 3 – 5 clones for 6 samples, 2 clones for 4 samples, and one clone for 2 samples. In addition, 5 clones from the coproculture of a chimpanzee's fecal sample were sequenced.

Forty-one newly obtained *cox-1* sequences of 849-bp length, coding 283 amino acids (AA), showed 25 different sequences due to nucleotide variations at 68 positions; 6 positions for the first nucleotide of the codon, 5 positions for the second, and 57 positions for the third. Using the first and second nucleotides for each codon of the *cox-1* gene, 12 haplotypes of 597-bp length were determined and their relationships were analyzed along with 2 haplotypes of *O. dentatum* and a single haplotype of *O. quadrispinulatum* (Table 4; Fig. 1A). A part of coprocultured larvae from a chimpanzee showed an identical haplotype with coprocultured larvae from three fecal samples from gorillas. In order to include more sequences from the two latter species, another shorter *cox-1* fragment of 392-bp length from the three *Oesophagostomum* spp. was retrieved from the DDBJ/EMBL/GenBank databases and trimmed by removing the third nucleotide for each codon; 4 haplotypes of *O. dentatum* (20 sequences) and 3 haplotypes of *O. quadrispinulatum* (6 sequences). Coprocultured

Table 3. Nucleotide variation in the ITS1 and 5.8S (A) and ITS2 (B) regions of rDNA of *Oesophagostomum stephanostomum* at MDNP, Gabon, and *Oesophagostomum bifurcum*

(A) ITS1 and 5.8S rDNA

Species	Locality	Host (No. of fecal samples)	No. of clones	DDBJ/EMBL/GenBank accession no.	Reference	ITS1*							5.8S*		
						6/7	7	14	137	138	202-204	233	76	83	91
O. stephanostomum	MDNP, Gabon	Gorillas (18) and chimpanzee (1)	42	AB821013-AB821017	The present study	-	A	C	C	C	GCC	T	A	A	A
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821018	The present study	·	·	·	·	·	· · ·	·	·	·	G
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821019	The present study	·	·	·	·	·	· · ·	·	G	·	·
O. stephanostomum	MDNP, Gabon	Gorillas (6)	8	AB821020, AB821021	The present study	C	C	·	·	·	· · ·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821022	The present study	C	C	·	·	·	· · ·	·	·	G	·
O. stephanostomum	MDNP, Gabon	Gorillas (3)	4	AB821023, AB821024	The present study	·	·	G	·	·	· · ·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1) and chimpanzee (1)	3	AB821025, AB821026	The present study	T	·	·	·	·	· · ·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821027	The present study	·	·	G	·	·	· · ·	A	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821028	The present study	·	·	·	·	·	- - -	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821029	The present study	·	·	·	T	T	· · ·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821030	The present study	T	·	·	T	T	· · ·	·	·	·	·

(B) ITS2

Species	Locality	Host (No. of fecal samples)	No. of clones	DDBJ/EMBL/GenBank accession no.	Reference	ITS2*																	
						34	54	62	82	100	105	111	118	122	123	137	162	165	168	179	182	203
O. stephanostomum	MDNP, Gabon	Gorillas (20) and chimpanzee (1)	61	AB821013, AB821017-AB821021, AB821023, AB821024, AB821026-AB821030	The present study	G	T	G	G	T	C	G	A	A	G	A	C	C	C	T	G	C
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821015	The present study	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	2	AB821014, AB821025	The present study	A	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821016	The present study	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·
O. stephanostomum	MDNP, Gabon	Chimpanzee (1)	1	AB821022	The present study	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·
O. stephanostomum	Kibalek, Uganda	Chimpanzee (8), blue monkeys (5), black and white colobus (2), gray-cheeked mangabey (2), red colobus (3), and red-tailed quenon (12)	32	KF250585-KF250588, KF250592, KF250594, KF250637-KF250644, KF250647, KF250648**	Ghai *et al.*, 2014	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
O. stephanostomum	Tanzania	Chimpanzee (1)	3	AF136576***	Gasser *et al.*, 1999b	·	·	·	·	·	·	·	·	W	·	·	·	·	·	·	R	M
O. bifurcum	Togo	Human (1)	1	Y11733***	Ob10; Romstad *et al.*, 1997	A	·	R	A	G	Y	R	R	T	R	·	T	·	Y	A	·	·
O. bifurcum	Togo	Human (2)	2	Y11733***	Ob11, Ob14; Romstad *et al.*, 1997	A	·	M	A	G	T	R	·	T	·	·	T	·	·	A	·	·
O. bifurcum	Togo	Human (1)	1	Y11733***	Ob12; Romstad *et al.*, 1997	A	·	A	A	G	Y	R	R	T	R	·	T	·	·	A	·	·
O. bifurcum	Ghana and Togo	Human and Mona monkey	9	AF136575***	T1; Gasser *et al.*, 1999a	A	·	A	A	G	T	A	·	T	·	·	T	·	·	A	·	·
O. bifurcum	Ghana	Human	3	AF136575***	T3; Gasser *et al.*, 1999a	A	·	R	A	G	T	A	·	T	R	·	T	·	·	A	·	·
O. bifurcum	Ghana and Togo	Human	1	AF136575***	T4; Gasser *et al.*, 1999a	A	·	C	A	G	T	A	·	T	·	·	T	·	·	A	·	·
O. bifurcum	Ghana	Mona monkey	1	AF136575***	T2; Gasser *et al.*, 1999a	A	·	·	A	G	·	·	·	T	R	·	T	·	·	A	·	·
O. bifurcum	Togo	Human	2	AF136575***	T5; Gasser *et al.*, 1999a	A	·	·	A	G	·	·	G	T	A	·	T	·	·	A	·	·
O. bifurcum	Togo	Human	1	AF136575***	T6; Gasser *et al.*, 1999a	A	Y	·	A	G	Y	·	R	T	·	·	T	·	·	A	·	·
O. bifurcum	Togo	Human	9	AF136575***	T7; Gasser *et al.*, 1999a	A	·	·	A	G	Y	R	R	T	R	·	T	·	·	A	·	·
O. bifurcum	Togo	Human	1	AF136575***	T8; Gasser *et al.*, 1999a	A	Y	M	A	G	T	A	·	T	·	·	T	·	Y	A	·	·
O. bifurcum	Togo	Human	1	AF136575***	T9; Gasser *et al.*, 1999a	A	·	·	A	G	Y	·	R	T	R	·	T	·	·	A	·	·

* Nucleotide position is expressed relative to the 5'-terminus of each region of *O. stephanostomum* (DDBJ/EMBL/GenBank accession no. AB821013). Dots denote an identical base to that of the uppermost sequences, and gaps are indicated by '-'

** There are additional sequences such as KF250595, KF250598, KF250599, KF250605, KF250606, KF250612-KF250619, KF250631, KF250634, KF250635 and KF250653

*** Representative sequence deposited by the original authors

Table 4. Amino acid (the first and second nucleotides of a codon) variation in a partial cox-1 region of mtDNA of *Oesophagostomum stephanostomum* at MDNP, Gabon, and two other *Oesophagostomum* spp.

Species	Haplotype	DDBJ/EMBL/GenBank accession no.	Host (No. of fecal samples)	Reference	No. of clones	Amino acid (AA) position*																				
						8	35	37	43	44	54	66	90	113	125	187	188	189	194	204	205	230	242	244	275	279
O. stephanostomum	S1	AB821032	Gorillas (6)	The present study	15	Asp (GAU)	Val (GUU)	Met (AUA)	Trp (UGA)	Thr (ACU)	His (CAY)	Leu (CUU)	Ser (AGA)	Leu (UUR)	Leu (UUR)	Leu (CUR)	Gly (GGU)	Met (AUR)	Leu (UUR)	Trp (UGG)	Ala (GCY)	Val (GUS)	Thr (ACW)	Phe (UUU)	Ser (UCU)	Leu (UUR)
O. stephanostomum	S2	AB821033	Gorilla (1)	The present study	2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Leu (CUG)
O. stephanostomum	S3	AB821034; AB821035	Gorillas (3) and chimpanzee (1)	The present study	9	·	·	·	·	·	·	·	·	Leu (CUA)	·	·	·	·	·	·	·	·	·	·	·	·
O. stephanostomum	S4	AB821036	Gorilla (1)	The present study	1	·	·	·	·	·	·	·	·	Leu (CUA)	·	Leu (UUA)	·	·	·	·	·	·	·	·	·	·
O. stephanostomum	S5	AB821037	Gorilla (1)	The present study	1	·	·	·	·	·	·	·	·	Leu (CUA)	·	·	·	·	·	·	·	·	·	Tyr (UAU)	·	·
O. stephanostomum	S6	AB821038	Gorilla (1)	The present study	1	·	·	·	·	·	·	·	·	Leu (CUA)	·	·	·	·	·	·	·	·	·	·	Pro (CCU)	·
O. stephanostomum	S7	AB821039	Gorilla (1)	The present study	1	·	·	·	·	Ala (GCU)	·	·	·	Leu (CUA)	·	·	·	·	·	·	·	·	·	·	·	·
O. stephanostomum	S8	AB821040	Gorilla (1)	The present study	1	·	·	·	Arg (CGA)	·	·	·	Thr (ACA)	·	·	·	·	·	·	·	·	·	·	·	·	·
O. stephanostomum	S9	AB821041	Gorilla (1)	The present study	2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Met (AUG)	·	·	·	·
O. stephanostomum	S10	AB821042	Gorilla (1)	The present study	5	·	·	Thr (ACA)	·	·	·	·	·	Leu (CUA)	·	·	·	·	·	·	Asp (GAU)	·	·	·	·	·
O. stephanostomum	S11	AB821043	Gorilla (1)	The present study	1	·	·	Thr (ACA)	·	·	·	·	·	·	·	·	·	·	·	Ter (UAG)	·	·	·	·	·	·
O. stephanostomum	S12	AB821044	Chimpanzee (1)	The present study	2	·	·	·	·	·	Arg (CGC)	·		Leu (CUA)	·	·	·	Thr (ACG)	·	·	·	·	·	·	·	·
O. dentatum	D1	GQ888716	An experimentally infected pig in Denmark	Jex et al., 2010	—	·	·	·	·	·	·	Leu (UUA)	·	·	Leu (CUG)	Leu (UUG)	·	·	Leu (CUA)	·	·	·	·	·	·	·
O. dentatum	D4	FM161882	A slaughtered pig in China	Lin et al., 2012a	—	·	·	·	·	·	·	Leu (UUA)	·	·	·	Leu (UUG)	Asp (GAU)	·	·	·	·	·	·	·	·	·
O. quadrispinulatum	Q2	FM161883	A slaughtered pig in China	Lin et al., 2012a	—	Asn (AAU)	Ala (GCU)	·	·	·	·	Leu (UUA)	·	·	Leu (CUU)	Leu (UUA)	·	·	·	·	·	·	Ala (GCU)	·	·	·

* Nucleotide position is expressed relative to the 5'-terminal of each region of *O. stephanostomum* (DDBJ/EMBL/GenBank accession no. AB821032). Dots denote an identical base to that of the uppermost sequence. Distinct AA from the uppermost sequence is written in squares

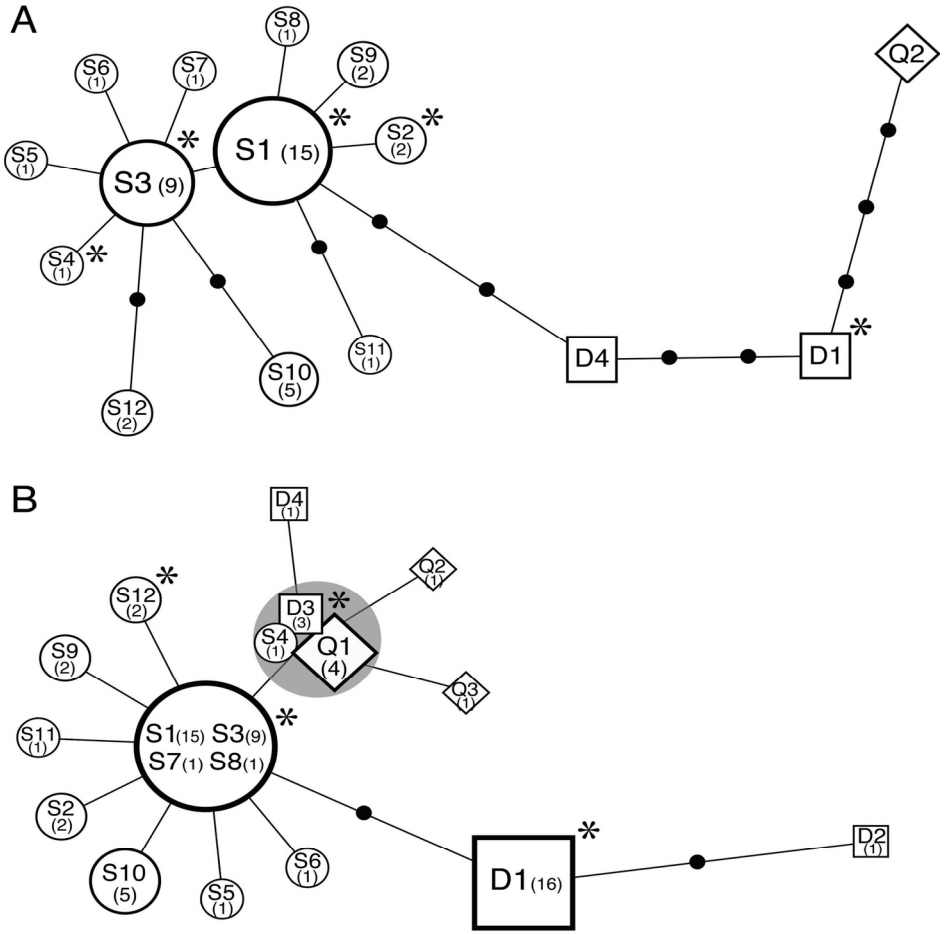

Fig. 1. Relationships of *cox-1* haplotypes of three *Oesophagostomum* spp. based on trimmed 597-bp long sequences (A) and trimmed 262-bp long sequences (B). *O. stephanostomum*, ○ (S1–S12 haplotypes); *O. dentatum*, □ (D1–D4 haplotypes); and *O. quadrispinulatum*, ◇ (Q1–Q3 haplotypes). Numbers in parentheses after the haplotype indicate the number of clones. Asterisks denote an identical AA sequence shared by different haplotypes.

Oesophagostomum larvae examined in the present study showed 9 haplotypes (41 sequences) (Fig. 1B). Haplotypes of each *Oesophagostomum* sp. were closely related, although *O. dentatum* showed two groups of haplotypes with at least three nucleotide differences through trimmed sequences of 262-bp length. Irrespective of such a higher number of nucleotide variations, 27 clones had the same AA sequence and the remaining 14 clones had eight different AA sequences (Table 4).

Discussion

In the present study, coprology and coproculture were applied to survey helminth infection of western lowland gorillas at MDNP, Gabon, and villagers living near MDNP. Although strongylid infection was the most prevalent in both gorillas and villagers, gorillas were infected with *O. stephanostomum* and hookworms, whereas villagers were infected solely with hookworms. *Oesophagostomum* spp. are the most common nematode of Old World monkeys and apes, including lowland gorillas. Freeman *et al.* (2004) reported a high prevalence (100 %) of strongyles / trichostrongyles for their samples from western lowland gorillas at Bai Hokou, Central African Republic. From non-invasive parasitological surveys of chimpanzees and seven other non-human primate species at Kibale National Park, Uganda, high prevalences of *Oesophagostomum* eggs were observed (8.3 – 85 %) (Krief *et al.*, 2005; Bezjian *et al.*, 2008; Ghai et al., 2014). From similar surveys of chimpanzees in Gambo Stream and Mahale National Parks, Tanzania, fairly high prevalences of *Oesophagotomus* eggs were recorded (38 – 91 %) (File *et al.*, 1976; Huffman *et al.*, 1996, 1997). For *Oesophagostomum* samples collected from Mahale chimpanzees, the species identification as *O. stephanostomum* had been made based on morphology of expelled adult worms in the feces after natural medical plant use of the primate, as well as coprocultured larvae (Huffman *et al.*, 1996, Huffman & Caton, 2001).

Recent molecular genetic studies have demonstrated that the ITS2 nucleotide sequence of rDNA allows an unequivocal identification of a range of strongylid nematode species, irrespective of the developmental stage of parasites. Based on this molecular strategy of species identification, we identified the prevalent helminth parasite of

western lowland gorillas at MDNP as *O. stephanostomum*, in addition to the *Oesophagostomum* morphology of coprocultured L3 larvae. Prior to the present study, a single ITS2 nucleotide sequence of *O. stephanostomum* was deposited in the DDBJ/EMBL/GenBank databases (accession no. AF136576). This sequence was based on three worms collected from a chimpanzee in Tanzania (Gasser *et al.*, 1999b), and it contained polymorphic bases at just three positions throughout its entire length. Furthermore, our preliminary direct sequencing of the ITS1–5.8S–ITS2 rDNA of seven coproculture samples generated two relatively clear forward sequences and five problematic forward sequences due to overlapped nucleotides by reading slips for all lengths, whereas all reverse sequences were relatively clear (unpublished data). Based on these findings, we assumed the presence of genetic variations in the ITS region and attempted to clarify the range and rate of nucleotide sequence diversity of *O. stephanostomum* found in gorillas at MDNP using the technique of DNA cloning. As shown in Table 3, a nucleotide insertion at a position between 6 and 7 of ITS1, which was found at a frequency of 13/68 (19.1 %), could cause unsuccessful direct forward sequencing of ITS1–5.8S–ITS2 of the parasite. It is not clear whether the observed variations were intraindividual or interindividual ones, because we extracted parasite DNA from 4 – 34 worms for PCR amplification of the ITS1–5.8S–ITS2 region. Nevertheless, the ITS region of *O. stephanostomum* at MDNP was variable to some extent; 1 – 3 nucleotide variations occurred in the ITS1 region at a rate of 35.3 % and 1 – 2 nucleotide variations occurred in the ITS2 region at a rate of 10.3 %. In contrast to our study, the quite recent work by Ghai *et al.* (2014) found, however, no nucleotide variations or polymorphic nucleotide sites in the ITS2 region of *O. stephanostomum* collected from 8 chimpanzees, 5 blue monkeys, 2 black and white colobus, 2 gray-cheeked mangabey, 3 red colobus and 12 red-tailed quenon at Kibale National Park, Uganda, and all sequences deposited by them to the DDBJ/EMBL/GenBank databases are similar to the most prominent *O. stephanostomum* ITS2 sequence in western lowland gorillas in our study (see Table 3).

Nucleotide sequences of the partial *cox-1* gene, 849 bp in length, showed a high number of variations, particularly at the third nucleotide of codons. Indeed, substitutions of the first and second nucleotides of codons were quite limited (16.2 % of all substitutions). Consequently, despite a high number of genetic variations, AA sequences of the parasites were well conserved (Table 4). Regarding the 113th AA (Leu), two alternative codon usages of *O. stephanostomum* in gorillas at MDNP, i.e. UUR and CUA, were found. However, as with other different substitutions of a minority of clones, its significance with respect to population genetics is currently unknown. In the case of *O. dentatum*, we found that four haplotypes appeared to be divided into two groups (Fig. 1B). Although this segregation has no geographical distinction (Lin *et al.*, 2012b**)**, it is worth pursuing the background of this genetic divergence through the collection of more parasite samples from across the world. In contrast to these nucleotide divergences within species or between species, we found that AA sequences were often shared by different species or were different even within species (Table 4). There was no relationship between *O. bifurcum cox-1* haplotype groupings and the specific primate host infected (de Gruijter *et al.*, 2002), in contrast to the genetic segregation of this species to each host species, as demonstrated by amplified fragment length polymorphism and random amplified polymorphic DNA analyses (de Gruijter *et al.*, 2004, 2005; reviewed by Gasser *et al.*, 2006, 2009). Species identification or phylogenetic study of *Oesophagostomum* spp. using *cox-1* sequences should therefore be conducted with care.

Based on our genetic analyses of the ITS region of rDNA and *cox-1* gene of mtDNA, it is concluded that at MDNP, Gabon, only a single population of *O. stephanostomum* with notable molecular variations is prevalent in western lowland gorillas and probably chimpanzees, without zoonotic complication in local inhabitants. The possible genetic variations of the ITS region of rDNA and *cox-1*gene of mtDNA presented here may be valuable when only a limited amount of material is available for genetic analyses for *O. stephanostomum* species diagnosis. Furthermore, different spectra of minor genetic variations of such gene sequences might be seen at different localities, since polymorphic nucleotide sites are different between *O. stephanostomum* collected in Tanzania, Uganda and Gabon (Gasser *et al.*, 1999b; Ghai *et al.*, 2014; the present study). Or, as having been demonstrated by Ghai *et al.* (2014), genetic approaches can definitely find cryptic *Oesophagostomum* spp. For the morphological characterization of such new genotype(s) of the species, we might use living adult nematodes expelled by apes in the process of self-medication, i.e. leaf-swallowing, as was done previously (Huffman *et al.*, 1996, 1997; Huffman & Caton, 2001).

Acknowledgments

We acknowledge Le Centre National de la Recherche Scientifique et Technologique (CENAREST) and L'Institut de Recherches en Ecologie Tropicale (IRET) for permission and support to conduct the study in Gabon. We are indebted to villagers at MDNP for their understandings and supports to our works. This study was supported in part by the Global Environment Research Fund (GERF-061) from the Ministry of the Environment, Japan, and JSPS KAKENHI grants. We also acknowledge two anonymous reviewers of an earlier draft of the manuscript for their invaluable suggestions and insights. The authors declare they have no conflict of interest.

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RECEIVED APRIL 8, 2014

ACCEPTED MAY 14, 2014