1 **Molecular features of hookworm larvae(*Necator* spp.) raised by coproculture**

2 **from Ugandan chimpanzees and Gabonese gorillas and humans**

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4 Hideo Hasegawa^{a*}, Miho Shigyo^a, Yuka Yanai^a, Matthew R. McLennan^b, Shiho Fujita^c,

5 Patrice Makouloutou^{d, e}, Sayaka Tsuchida^f, Chieko Ando^{g, h}, Hiroshi Sato^d, Michael A.

6 Huffmanⁱ

7 ^aDepartment of Biology, Faculty of Medicine, Oita University, Hasama, Yufu, 879-5593,

8 Japan.

9 ^bFaculty of Humanities and Social Sciences, Anthropology Centre for Conservation,

10 Environment and Development, Oxford Brookes University, Gipsy Lane Campus,

11 Oxford OX3 0BP, UK

12 ^cDepartment of Basic Veterinary Medicine, Joint Faculty of Veterinary Medicine,

13 Kagoshima University, Kagoshima, Japan;

14 ^dLaboratory of Parasitology, Joint Faculty of Veterinary Medicine, Yamaguchi

15 University, Yamaguchi, Japan

16 ^eInstitute de Recherche en Ecologie Tropicale (IRET/CENAREST), BP 13354,

17 Libreville, Gabon

18 ^fGraduate School of Life and Environmental Sciences, Kyoto Prefectural University,

19 Shimogamo, Kyoto 606-8522, Japan

20 ^gGraduate School of Science, Kyoto University, Kyoto 606-8502, Japan

21 ^hGeneral Incorporated Association ECO-LOGIC, Oshikakubo, Fujinomiya, Shizuoka

22 419-0303, Japan

23 ⁱ Section of Social Systems Evolution, Primate Research Institute, Kyoto University,

24 41-2, Kanrin, Inuyama, Aichi, 484-8506 Japan

25 * Corresponding author at: Department of Infectious Disease Control, Faculty of

26 Medicine, Oita University, Hasama, Yufu, 879-5593, Japan. Tel.: +81 97 586 5702; fax:

27 + 81 97 586 5702.

28 *E-mail address:* hasegawa@oita-u.ac.jp (H. Hasegawa)

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30 **ABSTRACT**

31 Species composition of *Necator* hookworms was surveyed in (i) Ugandan
32 chimpanzees living around farms and villages at Bulindi, (ii) Gabonese gorillas under
33 habituation in Moukalaba-Doudou National Park (MDNP), and (iii) Gabonese
34 villagers living adjacent to MDNP. Internal transcribed spacers (ITS) of rDNA and
35 partial cytochrome oxidase subunit 1 (*CoxI*) gene of mtDNA were analyzed from
36 larvae obtained by coproculture. Three ITS types (I, II and III) and three *CoxI*
37 haplotype groups (A, B and C) were demonstrated. ITS type I and *CoxI* haplotype group
38 A, representing *Necator americanus*, were demonstrated in the hookworm larvae from
39 Gabonese gorillas and humans, but not from Ugandan chimpanzees. Type II and
40 haplotype groups B and C, presumably representing *N. gorillae*, were found in larvae
41 from Ugandan chimpanzees and Gabonese gorillas and humans. These features were
42 overall similar with those found previously in the Central African Republic. Meanwhile,
43 type III was proven in a larva from a Gabonese gorilla as the first demonstration from a
44 non-human primate. *CoxI* haplotypes obtained from Ugandan chimpanzees formed a
45 subgroup within group B, presumably reflecting dispersal and diversification processes
46 of the apes.

47 Keywords: *Necator*, great apes, humans, Gabon, Uganda, DNA sequencing

48

49 Hookworms are major blood-sucking nematode parasites of humans and other
50 mammals causing anemia that is sometimes fatal [1]. It is estimated that 740 million
51 people are infected globally, including a high prevalence in sub-Saharan Africa
52 [1]. *Necator americanus* is the predominant species of hookworm infecting humans today
53 [1]. Besides humans, *Necator* hookworms are also known to infect some non-human
54 primates including great apes, and some non-primate mammals [2]. All great ape species
55 and subspecies are classified as Endangered or Critically Endangered [3]. However,
56 they increasingly inhabit disturbed environments in close proximity to humans [4]. The
57 infective larvae of *Necator* infect by skin penetration when a host walks on
58 contaminated soil or through grasses to which the larvae are clinging [1]. Thus, enhanced
59 conditions for zoonotic transmission of *Necator* have potential consequences for both
60 great ape conservation and public health.

61 In 2014, it was proven that *N. americanus* and a second species, referred to
62 as '*Necator* sp.', are shared by humans and western lowland gorillas (*Gorilla gorilla*
63 *gorilla*) in tropical rainforest in the Dzanga-Sangha Protected Areas (DSPAs), Central
64 African Republic (CAR) [2]. Recently, morphological study of hookworms expelled
65 from researchers, who had participated in gorilla surveys in DSPAs, demonstrated
66 *Necator gorillae* besides *N. americanus* [5], though molecular comparisons of adult
67 worms are still underway. It was strongly suggested that the great apes could be a
68 reservoir of *Necator* hookworms for humans and *vice versa* in DSPAs [2]. Presumably, a
69 similar situation may occur in other African sites, where humans and great apes share
70 habitats. Although undetermined *Necator* species have been demonstrated by DNA

71 analysis from chimpanzees at Kibale National Park, Uganda[6,7], and bonobos in
72 Bolobo Territory, Democratic Republic of Congo [8], transmission to humans has not yet
73 been proven. In this study, we aimed to identify the species composition of
74 *Necator* hookworms among East African chimpanzees (*Pan troglodytes schweinfurthii*)
75 in Bulindi, Uganda, and western lowland gorillas in Moukalaba-Doudou National Park
76 (MDNP), Gabon. Nineteen human inhabitants living adjacent to MDNP were also
77 examined; however, it was not possible to sample humans from Bulindi in the present
78 study.

79 Bulindi (1°28'N, 31°28'E) is situated in a region of notably close spatial
80 overlap and contact between wild chimpanzees and local humans [9,10]. Here,
81 chimpanzees inhabit small, degraded forest fragments amid farmland and villages
82 [11,12]. The study group comprised 19 individuals during the sampling period. They are
83 sympatric with four other diurnal non-human primates: olive baboons (*Papio anubis*),
84 tantalus monkeys (*Chlorocebus tantalus*), black and white colobus monkeys (*Colobus*
85 *guereza*) and blue monkeys (*Cercopithecus mitis*).

86 MDNP (2°26'S, 10°25'E) is composed of a mosaic of forest, savanna, and
87 swamp. It is inhabited by western lowland gorillas and central African chimpanzees (*P.t.*
88 *troglodytes*). A habituation program for one group of gorillas, numbering 20 individuals
89 during the sampling period, has been carried out since 2005[13]. The Moukalaba River
90 provides a natural barrier between local villages and the home ranges of great apes.
91 Besides great apes, nine species of primates, i.e., mandrill (*Mandrillus sphinx*),
92 grey-cheeked mangabey (*Lophocebus albigena*), northern

93 talapoin(*Miopithecusogouensis*), red-capped mangabey (*Cercocebus**torquatus*),
94 crowned monkey (*Cercopithecuspogonias*), putty-nosed monkey
95 (*Cercopithecus**nictitans*), moustached monkey (*Cercopithecus**cephus*), elegant
96 needle-clawed galago (*Euoticuselegantulus*) and lesser galagos (*Galagospp.*),inhabit the
97 park [14].

98 Fecal samples from great apes were collected non-invasivelyas reported
99 previously for Bulindi[15] and MDNP [16]. Fecal samples from farmersof the western
100 Bantu language group living near MDNP were also obtained [16]. They had no direct
101 contact with gorillas, but often entered the forest[17].All feces collected were subjected
102 to the modified Harada-Mori filter paper culture [18]. After 7–14 days, the bottom water
103 was checked with a magnifying glass, and larva-positive water was transferred to a 5
104 mL serum tube using a disposable plastic pipette, fixed and stored in >99% ethanol in
105 Uganda [15]and 80% ethanol in Gabon [16].

106 Methods of DNA extraction, amplification and sequencing followed those
107 employed previously [2,15].Internal transcribed spacers (ITS) of rDNA and partial
108 cytochrome *cox*idase subunit 1 (*CoxI*) gene of mtDNAwere sequenced.Types of ITS
109 and *CoxI* sequences were determined according to those reported previously [2].
110 Phylogenetic analysis was made for *CoxI* nucleotide and amino acid sequences by
111 neighbor-joining (NJ) and maximum-likelihood (ML) methods, using MEGA5 (v. 5.03)
112 [19] as employed previously [2,15].The nucleotide sequences determinedwere
113 registered in DDBJ/EMBL-Bank/GenBank with accession numbers LC088261
114 toLC088302 (rDNA and ITS) and LC088303 to LC088321 (*CoxI*).

115 Twenty-five (65.8%) of 38 cultures and 4 (22.2%) of 18 cultures made from
116 feces of chimpanzees in Bulindi and gorillas in MDNP, respectively, were positive for
117 *Necator* larvae. Eleven (57.9%) of 19 cultures made from feces of Gabonese villagers
118 were hookworm-positive [16], but only four *Necator*-positive cultures each with a few
119 larvae were available for the present study. Thirty-four larvae from Ugandan
120 chimpanzees and 23 larvae from Gabonese gorillas were selected randomly and subjected
121 to DNA sequencing (Table 1). Only three larvae from two Gabonese villagers responded
122 to DNA amplification (Table 1).

123 Sequencing was often difficult, especially in the middle area of ITS1 of type
124 II(=*Necator* sp.) where many repetitions of TG appear, giving incomplete sequences.
125 Full lengths of both ITS1 and ITS2 were successfully sequenced in only four and one
126 larvae from Ugandan chimpanzees and Gabonese gorillas, respectively. However,
127 typing of the incomplete sequences was possible based on characteristic indels (Table 1,
128 Supplemental Fig. 1). Only type II sequences were found from Ugandan chimpanzees,
129 whereas all three types were demonstrated from Gabonese gorillas (Table 1). This is the
130 first record of type III (also assigned to *Necator* sp. [2]) from a non-human primate.
131 Types I (= *N. americanus*) and II sequences were found in the larvae from Gabonese
132 humans (Table 1). Type I sequence obtained from one Gabonese gorilla was identical
133 with those reported previously from DSPA humans and gorillas (Supplemental Fig. 1,
134 2). Type II sequences obtained were mostly identical with that reported from DSPA [2],
135 but some had a few substitutions (Supplemental Fig. 1). Type III sequence differed
136 slightly from those reported in DSPA by having one to three substitutions

137 (Supplemental Fig. 1).

138 The larvae from Gabonese gorillas reacted effectively to the primers tested for
139 *CoxI* amplification, and gave sequences with 603 bp or more (Table 1). Meanwhile,
140 most larvae from Bulindichimpanzees did not respond to the primers, and only two
141 short sequences with 245bp and 589bp, respectively, were obtained (Table 1). NJ
142 analyses on the two sets of *CoxI* sequences each with 254 and 589 bp, respectively,
143 resulted in phylogenetic trees of largely similar topology, giving three clear haplotype
144 groups (Fig. 1). Isolates from Gabonese gorillas were scattered in the three groups. The
145 isolates from Ugandan chimpanzees were in group B, but diverged at a more basal
146 position than those of Gabonese and DSPA isolates. Presumably, chimpanzee-parasitic
147 *Necator* sp. in East Africa forms a subgroup, reflecting dispersal and diversification
148 processes of the apes. NJ and ML analyses on amino acid converted sequences from the
149 longer sequences did not give trees with branch bootstrap support within *Necator* spp.
150 above 52%. Among 11 larvae in which both ITS and *CoxI* were successfully
151 sequenced, only three combinations, I-A, II-B and II-C, were demonstrated (Table 2).

152 Local villagers in Bulindi sometimes defecate outdoors at the edges of
153 agricultural fields and inside the forest [20]. Because the villagers are strongly
154 presumed to harbor *N. americanus*, the chimpanzees could be exposed to the hookworm
155 larvae, especially when foraging or travelling on agricultural land [9,11]. Hence, absence
156 of *N. americanus* in Bulindi samples was unexpected. Interestingly, *N. americanus* was
157 also not found from three chimpanzees examined in DSPA [2]. This may imply that
158 chimpanzees are less susceptible to *N. americanus* in natural environments.

159 *Necator* sp. (presumably *N. gorillae* [5]) has zoonotic potential because it was
160 demonstrated from humans, gorillas and chimpanzees in the present study, and in the
161 same host species in the previous study in DSPA[2]. In Bulindi, chimpanzees defecate in
162 agricultural fields and nearhuman homes when travelling between forest patches and
163 foraging on agricultural crops [20]. Bulindi villagers most likely also harbor this
164 hookworm species. However, extensive parasitological surveys of local people are
165 necessary to demonstrate this zoonotic infection.

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167

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169

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248

249 Figure Caption

250 Fig. 1. Evolutionary relationships of *Necator* isolates inferred by the neighbor-joining
251 method based on *CoxI* nucleotide sequences with 245 (A) and 589 (B) positions.
252 Central African isolates include those collected from European researchers, who
253 acquired the infection in DSPA, CAR, besides local inhabitants [2]. Bootstrap test was
254 performed by 1000 replicates, and values over 50% are shown above the branches.

Figure 1

Host
 ● Human
 ○ Chimpanzee
 ● Gorilla

Locality
 ■ Uganda
 ■ Gabon
 ■ Central Africa
 ■ China
 ■ Japan

Table 1

Table 1. Number of *Necator* larvae analyzed and their ITS and *Cox1* types.

Locality	Host	No. fecal cultures utilized	No. larvae analyzed	ITS1	ITS2	Both ITS1 & ITS2	ITS type			<i>Cox1</i>	<i>Cox1</i> haplo- type group		
							I	II	III		A	B	C
Bulindi, Uganda	Chimpan- zee	12	34	30 (7)*	30 (13)	26 (4)		34		2		2	
Moukalaba- Doudou, Gabon	Gorilla	5	23	14 (3)	3 (3)	3 (1)	3	10	1	17	5	6	6
	Human	2	3	3			2	1					

* Whole length sequenced.

Table 2

Table 2. Combination of ITS types and *Cox1* haplotype groups.

Locality	Host	No. larvae both ITS and <i>Cox1</i> were sequenced	ITS types	<i>Cox1</i> haplotype groups		
				A	B	C
Bulindi, Uganda	Chimpanzee	2	II		2	
Moukalaba-Doudou, Gabon	Gorilla	9	I	3		
			II		4	2

Necator americanus and *Necator* cf. *gorillae* are ‘shared’ by humans and great apes in African ecosystems

Graphical abstract

Phylogenetic tree on *Cox-1* nucleotide sequences