

Short Communication

Genetic variation of NADH dehydrogenase subunit 1 (*nad1*) mitochondrial gene sequence in adult *Necator americanus* hookworms recovered from a female patient in Thailand

Eamsobhana, P.^{1*}, Yong, H.S.², Roongruangchai, K.¹, Tungtrongchitr, A. and Wanachiwanawin, D.^{1*}

¹Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

²Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

*Corresponding author e-mails: praphathip.eam@mahidol.ac.th; darawan.wan@mahidol.ac.th

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Abstract. Two female and one male adult hookworms were recovered from a female patient in Thailand. Based on gross and microscopic morphology, the three hookworms are members of *Necator americanus*. Phylogenetic reconstruction based on partial NADH dehydrogenase subunit 1 (*nad1*) mitochondrial gene sequences shows that these hookworms belong to the same genetic lineage as *N. americanus* adult worm from Zhejiang, China. The male and female hookworms were genetically distinct, belonging to two different *nad1*-haplotypes. This is the first report targeting the *nad1* gene on the identification and genetic characterization of the human hookworms originated from infected patient. The *nad1* gene marker is useful for species and higher taxa differentiation of hookworms.

Hookworms are blood-feeding nematodes (roundworms) belonging to the family Ancylostomatidae. At least 68 hookworm species have been described in 9 orders, 24 families, and 111 species of wild mammals (Seguel & Gottdenker, 2017). Hookworms possess hooked mouthparts which enable them to attach to the intestinal wall, usually in the duodenum of their mammalian host, to rupture capillaries and feed on blood. Pathogenesis of hookworm disease is mainly a consequence of anemia due to blood loss during attachment and feeding of the hookworm in the intestine.

There are two human hookworm species (*Ancylostoma duodenale* and *Necator americanus*) and two zoonotic counterparts (*A. caninum* and *A. ceylanicum*) (Pullan *et al.*, 2014). In many developing countries, human hookworms are a leading cause of

iron deficiency anemia (Hotez *et al.*, 2005). Globally, an estimated 438.9 million people (prevalence of 7.8%) were infected with hookworm in 2010, with 281.8 million (prevalence of 7.5%) in Asia and 77.0 million in Southeast Asia (prevalence of 12.6%) (Pullan *et al.*, 2014). The World Health Organization (WHO) estimated that up to 600 million people are infected with hookworm worldwide (World Health Organization, 2015).

The national prevalence rate of hookworm infection in Thailand is 6.5% (Wongsaroj *et al.*, 2014). The prevalence rate in a locality may be as low as 1.75% in a rural community in Huay Muang village, located in Tha Wang Pha District, Nan Province, Northern Thailand (Chaisiri *et al.*, 2019) and 2.4% in Yo Island urban community, Songkhla, Southern Thailand

(Kitvatanachai *et al.*, 2019), to as high as 14.45% in Northeastern Thailand (Songserm *et al.*, 2012) and 28% in Northeastern Thailand (Triteeraprapab *et al.*, 1999).

N. americanus and *A. duodenale* are the two main hookworms infecting humans in Thailand (Phosuk *et al.*, 2013) with *N. americanus* as the most common hookworm (Anantaphruti *et al.*, 2002; Jiraanankul *et al.*, 2011). Molecular analysis, based on internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region of the nuclear ribosomal RNA gene, has been used to confirm human infections with *N. americanus*, *A. duodenale* and the animal hookworm *A. ceylanicum* (Jiraanankul *et al.*, 2011; Phosuk *et al.*, 2013). In addition to ITS gene, cytochrome *c* oxidase subunit I (*cox1*) mitochondrial gene has been used to identify hookworm species (Monteiroa *et al.*, 2019). Although species-specific molecular-based techniques targeting the *cox1* mitochondrial gene have received the widest applications to differentiate human hookworms from infected patients, studies based on sequences for more mitochondrial genes, in contrast to the commonly employed *cox1* gene sequences may further help us understand the genetic variation or genetic population structure of human hookworms that could provide better insight on the disease epidemiology. As far as we are aware, there is no report on the use of *nad1* (NADH dehydrogenase subunit 1) mitochondrial gene sequences for genetic differentiation of hookworm species. We report here the use of partial *nad1* gene sequences to identify and differentiate adult hookworms recovered from a female patient in central Thailand.

The present case with hookworm infection was a 76-year-old female patient who lived in Bang Bua Thong district, Nonthaburi Province, central Thailand. She presented with exertional dyspnea and abdominal discomfort for a week. The chest roentgenogram revealed no pulmonary congestion. Stool examination was positive for occult blood and hookworm eggs.

Gastroduodenoscopy found multiple duodenal erosions and numerous slender moving roundworms on the duodenal mucosa. Three worms were removed by endoscopic biopsy forceps and sent to the Department of

Parasitology, Faculty of Medicine Siriraj Hospital for worm identification.

The worms were washed extensively in physiological saline and identified to species by morphological characters (Rep, 1963). The three adult worms (female: 2, male: 1) were round-cylindrical in shape and the buccal cavity at the anterior end was characterized by one pair of cutting plates in the buccal capsule. The male worm measured 8 mm long and 0.3 mm wide, with prominent copulatory bursa at the posterior end, and a deep notch at the dorsal costa (Fig. 1a). The two female worms, without posterior copulatory bursa, measured around 9.5 mm long and 0.4 mm wide (Fig. 1b). Based on gross and microscopic morphology, the three hookworms are members of *N. americanus*. After gross examination, the worms were preserved in absolute ethanol and kept at -40°C for DNA extraction. The oligonucleotide primer pair (Forward: 5'-TTCTTATGAGATTGCTTTT-3' and Reverse: 5'-TATCATAACGAAAACGAGG-3') for NADH dehydrogenase subunit 1 (*nad1*) was used for polymerase chain reaction to amplify a fragment (~370 bp) of the mitochondrial *nad1* gene according to Luo *et al.* (2017) with slight modification. Genomic DNA was isolated from individual hookworm by using the fast technology for analysis of nucleic acid (FTA) classic card method (Whatman BioScience, Newton Center, Massachusetts, USA) and according to the instructions of the manufacturer. Captured nucleic acid on the FTA cards was purified and polymerase chain reaction (PCR) master mix was added directly to the DNA punch in a PCR tube. The PCR mixture was denatured at 94°C for 5 min, followed by 35-cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, followed by post-amplification at 72°C for 5 min. The sequencing procedure follows that previously described by Eamsobhana *et al.* (2010). The *nad1* sequences of other hookworm species available from GenBank were included in alignments for comparison. Phylogenetic trees were reconstructed by the Maximum Likelihood (ML) and Neighbor Joining methods using MEGA 6.0. Bootstrap value with 1000 replication was confirmed in term of reliability.



Figure 1. (a) Adult male and (b) female worms of *Necator americanus* removed from duodenum of the patient by gastroduodenoscopy; (c) Male and (d) female anterior extremity showing one pair of cutting plates in the buccal capsule; (e) Male posterior extremity showing copulatory bursa; (f) Female posterior extremity with anus near the rear end.

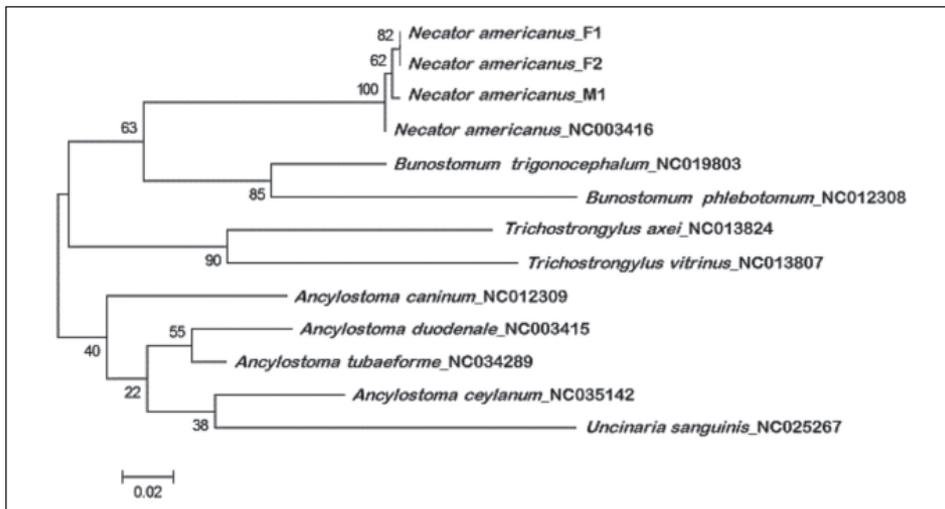


Figure 2. Phylogenetic tree of the *Necator americanus* adult hookworms (F1, F2 and M1) recovered from a female patient in Thailand and Ancylostomatidae taxa from GenBank (*Ancylostoma* spp., *Bunostomum* spp., *Uncinaria* sp.) based on partial *nad1* nucleotide sequences reconstructed by the Maximum Likelihood (ML) method using MEGA 6.0, with *Trichostrongylus* spp. as outgroup taxa. Identical tree was produced by Neighbor Joining (NJ) method.

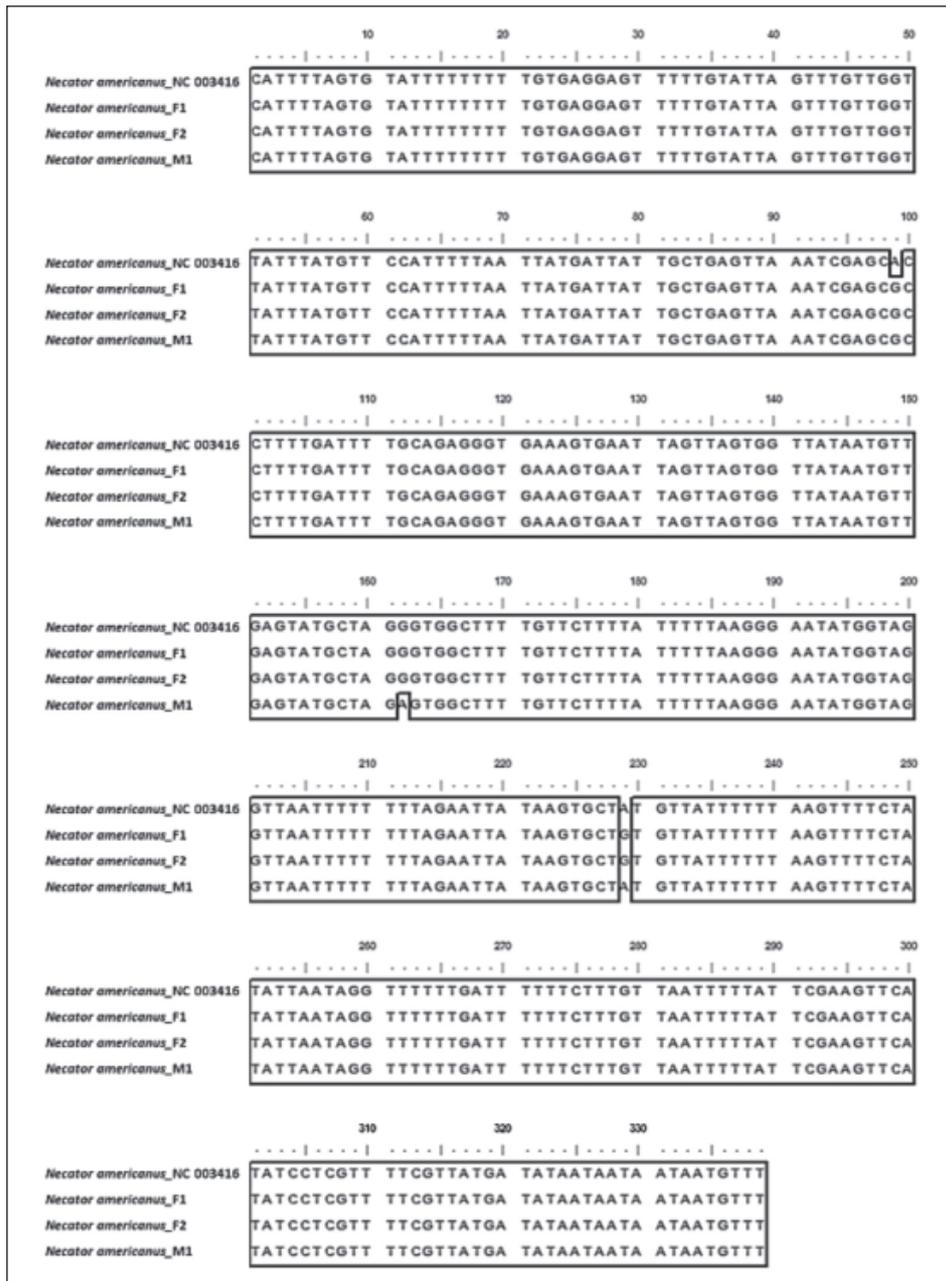


Figure 3. Comparison of 339 nucleotides of a portion of *nad1* mitochondrial gene between the Thai hookworms, *Necator americanus* (F1, F2 and M1) and *Necator americanus* (NC003416) from Zhejiang, China.

Phylogenetic reconstruction based on partial *nad1* sequences, with a final length of 339 bp, shows that these hookworms belong to the same genetic lineage as *N. americanus* adult worm from Zhejiang, China (Fig. 2), and are distinctly different from other hookworm taxa (*Ancylostoma* spp., *Bunostomum* spp.,

and *Uncinaria* sp.) (Fig. 2). The genus *Necator* forms a clade with the genus *Bunostomum*, while the genus *Ancylostoma* forms a clade with the genus *Uncinaria*.

Three positions (99, 162 and 229) in the 339 bp of *nad1* sequences of *N. americanus* show nucleotide variation (Fig. 3). The two

female hookworms from Thailand have identical partial *nad1* sequence. *N. americanus* from China is represented by nucleotide A at position 99 compared to G in the three Thailand sequences. The male hookworm from Thailand possesses nucleotide A at position 162 compared to G in the other hookworms. The two female hookworms from Thailand are represented by nucleotide G at position 229 compared to A in the male hookworm from Thailand and the hookworm from China.

The presence of two *nad1*-haplotypes in the three hookworms recovered from a female patient indicates possibility of high genetic diversity in *N. americanus* hookworm in Thailand. A high genetic diversity of 23 *cox1*-haplotypes has been reported for *N. americanus* in Brazil (Monteiroa *et al.*, 2019). Phylogeography study based on *nad1* gene sequences could add to our understanding of the genetic diversity of *N. americanus* hookworms in Thailand and other countries.

In summary, the present study, for the first time targeting the *nad1* gene reveals genetic variation in the three *N. americanus* hookworms recovered from a female patient in Thailand. The *nad1* gene marker will be useful for species and higher taxa differentiation of hookworms.

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