Molecular phylogenetic study of *Gongylonema* worms to understand interspecific borders and transmission dynamics in the natural environment

Gongylonema 属線虫の種区分と自然環境での伝播の理解を目指した分子系統学的研究

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CONTENTS

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ABSTRACT

Gongylonema spp. (Nematoda: Spirurida: Gongylonematidae) are thread-like spirurid nematodes dwelling in the mucosal epithelium of upper digestive tract of mammals and birds worldwide. *Gongylonema pulchrum* is well known as the gullet worm, and found in a wide spectrum of mammals such as cattle, sheep, goats, donkeys, cervids, equines, camels, bears, pigs, non-human primates, and human beings. Although generic diagnosis of the adult worms is not difficult due to the presence of characteristic verruciform protrusions in the anterior surface of body, it is hard to make a specific identification based solely on morphology due to distinct growth of the adult worms in different hosts. To overcome this difficulty in specific diagnosis, molecular genetic characterization of *Gongylonema* worms and clarification of intraspecific genetic variation or interspecific genetic differences are necessary. Previous studies in our laboratory provided long nucleotide sequences of ribosomal RNA gene (rDNA), including internal transcribed spacer (ITS) regions, and partial cytochrome *c* oxidase subunit 1 gene (*cox-1*) of Iranian and Japanese isolates of *G. pulchrum* from domestic or captive animals (cattle, and squirrel monkeys) and wild mammals (Japanese sika deer *Cervus nippon*, Japanese wild boars *Sus scrofa leucomystax*, and Japanese macaques *Macaca fuscata*), providing a spectrum of intraspecific genetic variation of *G. pulchrum* in Japan.

In Chapter I of the present study, I extended the research to rodent *Gongylonema* worms, i.e. *G. aegypti* from spiny mice *Acomys dimidiatus* in the Sinai Peninsula, Egypt, and *G. neoplasticum* from black rat *Rattus rattus* in Okinawa Island, Japan, to clarify interspecific genetic differences between them and *G. pulchrum*. This study finally disclosed the validity to differentiate *Gongylonema* worms from Nepalian water buffaloes *Bubalus bubalis* from *G. pulchrum* as an independent species, i.e. new species *G. nepalensis,* although adult worms of *G. pulchrum* and *G. nepalensis* showed an identical morphology except for different proportions of left spicule length against the body length (>24% vs. <22%, respectively). In addition, two *cox-1* haplotypes of *G. pulchrum* from cattle in Japan were also found in *G. pulchrum* worms collected from cattle in Alashan League, Inner Mongolia, China, suggesting that these two *cox-1* haplotypes might be widely distributed in East Asia. The origin of these two *cox-1* haplotypes of *G. pulchrum* in domestic or captive animals is unknown yet.

In Chapter II of the present study, I attempted to clarify genetic variation of *G. neoplasticum* from 127 rats of seven species (five *Rattus* spp., *Maxomys surifer*, and *Berylmys bowersi*) from Southeast Asia (Thailand, Cambodia, Laos, Philippines, and Indonesia), where originated the dominant hosts for the species worldwide, *Rattus norvegicus* (brown rats) and *Rattus rattus*. This study demonstrated substantial nucleotide variations of *G. neoplasticum* in the stomach mucosa of rats (24 haplotypes), and this data may facilitate the reliable specific differentiation of local rodent *Gongylonema* spp. from the cosmopolitan congener, *G. neoplasticum*.

In Chapter III of the present study, I characterized longer *cox-1* nucleotide sequences (852 bp) of *G. pulchrum* from wild mammals, such as sika deer, wild boars, Japanese macaques and feral alien Reeves's muntjacs *Muntiacus reevesi*, in Japan, and *G. nepalensis* from a red fox *Vulpes vulpes* and a wild boar *Sus scrofa meridionalis* on Sardiania Island, Italy, to clarify their haplotypes and relationships with the worms in domestic animals. *Gongylonema* worms from feral alien Reeves's muntjacs on Izu-oshima Island, Tokyo, showed *G. pulchrum* cattle *cox-1* haplotypes I and II, distinct from *cox-1* haplotypes of the worms from wild mammals in Japan. Genetic variation of *cox-1* nucleotide sequences of *G. nepalensis* from domestic and wild animals (*Bos Taurus*, *Ovis aries*, *Capra hircus*, *Ovis aries musimon*, *Vulpes vulpes*, and *Sus scrofa meridionalis*) on the Island was minimal, suggesting a shared transmission cycle among domestic and wild animals, which is distinct from separate transmission cycles between domestic and wild mammals, except for feral alien Reeves's muntjacs, at least in Japan.

I believe that my studies on *Gongylonema* worms mentioned above can provide a research platform for genetic differentiation of *Gongylonema* spp., followed by further characterization of different species of the genus. In addition, I have shown the utility of genetic characters of the worm in discussing transmission dynamics of the worms in nature.

General Introduction

The genus *Gongylonema* (Nematoda: Spirurida: Gongylonematidae) was erected by Raffaele Molin in 1857, when he described four species, i.e., *G. minimum* (type species) from a house mouse, *G. filiforme* from a monkey, *G. spirale* from deer, and *G. pulchrum* from a European wild boar (Molin 1857). From the present viewpoint, '*Spiroptera ursi*' described by Rudolphi (1819) from a European bear might be identical to *G. pulchrum* Molin, 1857 (Chandler, 1950), but his description was not satisfactory for specifying the species. Spirurid nematodes of the genus *Gongylonema* are very unique to have their adult-stage habitat in the stratified squamous epithelium of upper digestive tract such as gum, buccal mucosa, tongue, esophagus, and stomach (Fig. 1). The anterior part of body of *Gongylonema* worms is covered by the cuticle with numerous verruciform protrusions (Fig. 2). It is likely that this morphological feature makes the worm identification easier at least at the generic level, and numerous species, currently synonymized to *G. pulchrum* Molin, 1857, were described in the past as shown in Table 1. Isolation of the worm from a variety of host mammals of different classification categories such Carnivora (bears), Perissodactyla (equines), Cetartiodactyla (cattle, sheep, goats, donkeys, cervids, camels, and pigs), and Primates (non-human primates, and humans), and morphological variation particularly in worm length and specular length might support novel specific descriptions until 1930'. As introduced by Sato (2009), cross infection experiments by Ransom and Hall (1915, 1917), Baylis (1925), Schwartz and Lucker (1931), and Lucker (1932) demonstrated conspecificity of *G. pulchrum* from wild boars, *G. scutatum* from ruminants, and *G. ransomi* from pigs. Lichtenfels (1971) emphasized utility of relative length of *Gongylonema* worms to identify the species, instead of actually measured values of worms.

The pathogenicity of *G. pulchrum* is usually not high, and often parasitized host animals don't show any symptoms. When many worms infect, the surface of epithelium becomes rough and hemorrhaged with inflammation, then the hosts lose the appetite and the weight, or finally die in the severe cases. In the human cases, the patients claim intermittent yet persistent nausea, vomiting, or the sense of worms crawling under the oral mucosa. Often physicians suspect mental disorder in such patients (Wilde et al. 2001; Wilson et al. 2001; Hung et al. 2016; Libertin et al. 2017).

Life cycle of *Gongylonema* spp.

Female worm lays thick-shelled eggs containing a fully differentiated first-stage larva which has cephalic hooks and rows of minute spines around the rather blunt anterior end (Fig. 3). The tail of the first-stage larva is often blunt and surrounded by a circlet of minute spines. Eggs are excreted with feces and wait to be ingested by intermediate hosts. Intermediate hosts of *G. pulchrum* are dung beetles such as varied *Aphodius* spp. (*A. rectus*, *A. sordidius*, *A. elegans*, *A. haroldianus*, *A. urostigma*, *A. sumlimbatus*, *A. coloradensis*, *A. distinctus*, *A. femoralis*, *A. granaries*, *A. rubeolus*, and *A. vittatus*), *Blaps appendiculate*, *Liatongus phanaeoides*, *Caccobius jessonensis*, *Ontophagus bivertex minokuchians*, *Copris ochus*, and *Copris acutidens* (Alicata 1935, Kudo et al. 1996). Larvae of *G. pulchrum* are encysted in the hemocoel of the intermediate host (Alicata 1935). After death of insects in the water, the larvae are freed from the intermediate host, surviving in the water for two weeks in average, up to 34 days (Kudo et al. 1996). It means that the final host could be infected by drinking water containing the infective larvae (Alicata 1935; Cappucchi et al. 1982; Kudo et al. 1996).

G. pulchrum eggs hatched in the crop or gut of insects, and the larvae invaded the haemocoel within 24 hours (Alicata 1935). The first-stage larvae developed in the haemocoel or in other tissues such as muscles and adipose tissues, and underwent two molts. By approximately 19th day of infection, the second-stage larvae wandered in the hemocoel. The second molting occurred between 29 and 32 days after experimental infection, and the third-stage larvae are found after 32 days of infection (Ransom and Hall 1915; Lucker 1932; Alicata 1935; Anderson 1992). The second- and third-larval stages eventually become encapsulated. According to Alicata (1935), infective *G. pulchrum* larvae fed to guinea pigs excysted in the stomach and invaded the esophagus within one hour and half after feeding. At this time, the larvae migrated from the tissue of stomach to the esophagus through the junction of stomach and esophagus. Then the larvae migrated from esophagus to the wall of oral cavity such as tongue, palate or lining of a buccal cavity three days after experimental feeding, and after some growth there they returned to the esophagus.

Approximately 50 species have been described in the genus *Gongylonema* from birds and mammals on every continent except Antarctica since Rudolphi (1819), as written in Sato (2009), Kinsella et al. (2016) and Cordeiro et al. (2018).

Molecular genetic analyses to explore the parasite species and genetic groups

G. pulchrum has been recorded from a variety of mammals including humans worldwide. After successful cross infection experiments in the early 20th century (Ransom and Hall 1915; Baylis et al. 1926a, b; Schwartz and Lucker 1931; Lucker 1932), it is widely accepted that a single species '*G. pulchrum*' may be shared by multiple host mammalian species by ingestion of the intermediate host. Molecular genetic characterization of *G. pulchrum* was for the first

time reported by Halajian et al. (2010), followed by Makouloutou et al. (2013a, b). They analyzed nucleotide sequences of the ribosomal RNA gene (rDNA), including internal transcribed spacer (ITS) regions, and partial cytochrome *c* oxidase subunit 1 gene (*cox-1*) of Iranian and Japanese isolates of *G. pulchrum* from domestic or captive animals (cattle, and squirrel monkeys) and wild mammals (Japanese sika deer *Cervus nippon*, Japanese wild boars *Sus scrofa leucomystax*, and Japanese macaques *Macaca fuscata*), providing a spectrum of intraspecific genetic variation of *G. pulchrum* in Japan. At the same time, their studies dig up or recall several questions: 1) why domestic and wild animals have different genetic lineages of *G. pulchrum*?; 2) how wide distribution of detected genetic lineages of *G. pulchrum*?; 3) how genetically differentiated but morphologically similar *Gongylonema* worms should be specified?; etc. In most cases, taxonomic description based on morphological criteria has been made using a limited number of *Gongylonema* worms (Kinsella et al. 2016). Genetic validation of morphologically described *Gongylonema* worms might be necessary. In the present study, I have attempted to resolve taxonomic questions on *G. pulchrum* and additional mammalian *Gongylonema* spp. one by one.

Chapter I

Intraspecific and interspecific genetic variation of *Gongylonema pulchrum* and two rodent *Gongylonema* spp. (*G. aegypti* and *G. neoplasticum*), with the proposal of *G. nepalensis* n. sp. for the isolate in water buffaloes from Nepal

The work described in the chapter has been published as follows:

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Abstract

The gullet worm (*Gongylonema pulchrum*) has been recorded from a variety of mammals worldwide. In an earlier study, we demonstrated two separate transmission cycles in cattle (*Bos taurus*) and wild mammals in Japan based on nucleotide sequences of the ribosomal RNA gene (rDNA) and cytochrome *c* oxidase subunit I (*cox-1*) region of mitochondrial DNA of multiple isolates of different origins. Our earlier study additionally demonstrated two major *cox-1* haplotypes of *G. pulchrum* prevalent in cattle in Japan. In the present study, we collected *G. pulchrum* from cattle and goats (*Capra hircus*) in Alashan League, Inner Mongolia, China; *Gongylonema aegypti* from spiny mice (*Acomys dimidiatus*) in the Sinai Peninsula, Egypt; and *Gongylonema neoplasticum* from a black rat (*Rattus rattus*) in Okinawa Island, Japan, to analyze their genetic relationships with *G. pulchrum* in Japan. The gullet worms from Alashan League had almost identical rDNA nucleotide sequences and two *cox-1* haplotypes as seen in *G. pulchrum* from the cattle in Japan. The two rodent *Gongylonema* spp. had distinct rDNA nucleotide sequences compared with those of *G. pulchrum*; only the 18S and 5.8S rDNA sequences showed high identities at 97.2–98.7 %, while the remaining sequences were less than 75 % identical. The 18S, 5.8S, and 28S rDNA sequences of the two rodent *Gongylonema* spp. showed nucleotide identities of 99.8 % (1811/1814), 100 % (158/158), and 98.9 % (3550/3590), respectively. The *cox-1* regions showed 91.6% (338/369)–92.1% (340/369) identities, with completely identical amino acid sequences. The genetic diversities of three distinct *Gongylonema* spp. and their possible intraspecific genetic variation may allow us to resolve the taxonomic position of *Gongylonema* spp. which display few obvious morphological differences from their congeners. Consequently, the *Gongylonema* isolate from water buffaloes (*Bubalus*

bubalis) in Nepal reported in our previous study is concluded to be a new species, and *Gongylonema nepalensis* n. sp. is erected for it.

1. 1 Introduction

The gullet worm, *Gongylonema pulchrum* Molin, 1857, is a thread-like spirurid nematode found in the upper digestive tract of a variety of mammals worldwide (Lichtenfels 1971; Anderson 1992; Sato 2009). Due to an earlier trend when many species descriptions were based primarily on different isolation sources and/or some morphological uniqueness of observed worms, many *Gongylonema* spp. became junior synonyms of *G. pulchrum* based on cross-infection experiments (Ransom and Hall 1915; Baylis et al. 1926a, b; Schwartz and Lucker 1931; Lucker 1932) and meticulous morphological analyses (Schwartz and Lucker 1931; Lichtenfels 1971) as listed in Table 1. From the taxonomical viewpoint, the restriction of many taxa to *G. pulchrum* as synonyms is favorable. However, it should be noted that it does not mean that the same gullet worm lineages are shared widely by a variety of sympatric mammals through accidental ingestion of infected dung beetles or water contaminated with third stage larvae that have emerged from dead dung beetles (Kudo et al. 1996). Previous work from our laboratory (Makouloutou et al. 2013a) has demonstrated multiple origins, at least three, of *G. pulchrum* in Japan and their unique transmission cycles in domestic and wild mammals in the country. These findings were based on molecular genetic analyses of the ribosomal RNA gene (rDNA), particularly the internal transcribed spacer (ITS) regions, and partial cytochrome *c* oxidase subunit I (*cox-1*) region of mitochondrial DNA (mtDNA) of worms isolated from multiple mammalian hosts. The reason why two major *cox-1* haplotypes of *G. pulchrum* were found in cattle (*Bos taurus*), distinct from any haplotypes in wild mammals such as sika deer

(*Cervus nippon*), wild boars (*Sus scrofa leucomystax*), and macaque monkeys (*Macaca fuscata*), is unknown at present.

Most of the cattle in Japan are Japanese Black breed (beef cattle) and Holstein-Friesian breed (dairy cattle). The Japanese Black breed was recently established in the twentieth century by upgrading native Japanese cattle with various European breeds in several prefectures. Native Japanese cattle are speculated to have been introduced to Japan around the second century from northern China via the Korean Peninsula, accompanied by the introduction of rice cultivation (Mannen et al. 1998, 2004; Shi et al. 2004). Native Japanese cattle or native north-east Asian cattle (Turano-Mongolian type) might have a geographical variant of *G. pulchrum*, and the recent introduction of European breeds or Holstein-Friesian cattle to Japan might be another opportunity to disperse *G. pulchrum* of a distinct *cox-1* haplotype in the country.

To investigate such a hypothesis, we collected gullet worms from cattle in Inner Mongolia, China, and analyzed their nucleotide sequences of rDNA and the *cox-1* gene. In addition, we genetically characterized rodent *Gongylonema* spp., i.e., *Gongylonema aegypti* and *Gongylonema neoplasticum*, to try and understand more clearly the intraspecific variation of *G. pulchrum* from multiple mammalian hosts, as well as interspecific variation of *Gongylonema* spp.

1. 2 Materials and methods

1. 2. 1 Collection of parasites and morphological observation

Full-length esophagi of 68 cattle and 19 goats (*Capra hircus*) were collected in local places for livestock slaughter in Eerkehashiha, Alashan Left Banner, Alashan League, Inner Mongolia Autonomous Region, People's Republic of China (37°42′－38°38′ N, 103°21′－104°10′ E),

between 21 and 25 December 2010. These animals were bred and grazed on arid grasslands with some natural lakes, facing desert areas. In the laboratory of Animal Health Inspection, Hashkha, Alashan League, the esophagus was opened longitudinally and the mucosal layers were peeled from the underlying tissues. The mucosal surface was then carefully checked with the naked eye, and individual worms were carefully removed from the esophageal epithelium using fine forceps and fixed in 70 % alcohol or 10 % neutral-buffered formalin solution. The collected specimens, except for worms for DNA extraction, were deposited in the National Museum of Nature and Science, Tokyo, Japan, under specimen numbers NSMT-As3969–4022.

Three female worms of *G. aegypti* were collected from the stomach walls of three spiny mice (*Acomys dimidiatus*) from two wadis in the arid montane region of the southern Sinai Peninsula in Egypt (28°30′–40′N, 33°52′–57′E) (see 'Fig. 1' in Behnke et al. 2004) during October 2012. One female and two male worms of *G. neoplasticum* were collected from the stomach wall of a female black rat (*Rattus rattus*) trapped in Urazoe City, Okinawa Prefecture, Japan, on 10 December 2012 by Dr. Mikako Tamashiro, School of Health Sciences, University of the Ryukyu. The specimens of these two species were fixed in 70 % alcohol after collection.

1. 2. 2 Morphological examination of the gullet worm

Specimens fixed in 10 % neutral-buffered formalin solution were cleared by the addition of several drops of glycerol. Six female and six male adults from the cattle were observed under a light microscope and, figures were drawn with the aid of a camera lucida. Measurements were performed on these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) when necessary.

1. 2. 3 DNA extraction, polymerase chain reaction, and sequencing

Single worms fixed in 70 % alcohol were divided longitudinally into three equal parts. The middle part was washed three times in physiological saline and placed in a 1.5-ml plastic tube. The specimens in tubes were freeze-dried using a freeze dryer (Model EYELA FD-5N; Tokyo Rikakikai Co., Bunkyoku, Tokyo, Japan) and then crushed with individual clean plastic pestles. The parasite DNAs were extracted separately from these samples using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of overlapping rDNA fragments was performed in a 20-μl volume containing a DNA polymerase Blend Taq-Plus- (TOYOBO, Dojima Hama, Osaka, Japan) and universal eukaryotic primer pairs as previously described (Makouloutou et al. 2013a). PCR products for sequencing were purified using a FastGeneGel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan). Following direct sequencing of PCR amplicons, sequences were assembled manually with the aid of the CLUSTALW multiple alignment program (Thompson et al. 1994). For rDNA segments containing ITS1 and 2, the amplicon was cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO) and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.), and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers. The *cox-1* region of *G. pulchrum* mtDNA was amplified by two different primer pairs as follows: (1) BpCoxI-F1 and BpCoxIR1 as described in Makouloutou et al. (2013a) and (2) StrCoxAfrF (5′-GTG GTT TTG GTA ATT GAA TGG TT-3′) and MH28R (5′-CTA ACT ACA TAA TAA GTA TCA TG-3′) as described in Makouloutou et al. (2014). The amplicons were

sequenced after purification as described above. Nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank databases under the accession numbers LC026017– LC026049. Voucher specimens for these DNA analyses were deposited in the National Museum of Nature and Science, Tokyo, Japan, under specimen numbers NSMTAs4023–4035 (*G. pulchrum*), As4036–4038 (*G. aegypti*), and As4039 (*G. neoplasticum*).

1. 2. 4 Phylogenetic analysis

For phylogenetic analysis, the newly obtained *cox-1* sequences, 369 bp in length, of the *Gongylonema* worms collected in the present study and those of the same genus retrieved from the DDBJ/EMBL/GenBank databases were used. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the 'phylogeny.fr' website (http://www. phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

1. 3 Results

1. 3. 1 Prevalence and morphology of *G. pulchrum* in cattle and goats in Alashan League

The prevalences and intensities of adult *G. pulchrum* in the cattle and goats are shown in Table 2. From the cattle, an average of 5.3 worms (range 1–13) were collected, although 3–8 worms/host were most commonly encountered (55.1% of infected cases), followed by 1 or 2 worms/host (28.6 %) and more than 9 worms/host (16.3 %). From the goats, an average of 4.1 worms (range 1–7) were collected; 3–5 worms/goat was the most prevalent (66.7 %). The worms were embedded in a zig-zag pattern in the esophageal mucosa. Morphological features

including measurements of collected parasites were similar to those of *G. pulchrum* observed in cattle in Japan and other countries (Table 3).

1. 3. 2 rDNA of *G. pulchrum* in Alashan League, *G. aegypti* and *G. neoplasticum*

Approximately 6100-bp-long nucleotide sequences from the 5′-terminus of 18S to 28S rDNA of *G. pulchrum* from the cattle were obtained. These sequences contained 1782-bp long partial 18S rDNA, 378–391-bp-long ITS1, 158-bp-long 5.8S rDNA, 223–235-bp-long ITS2, and 3544-bp-long partial 28S rDNA. Nucleotide variations of each part of the rDNA were similar to those of the cattle-type *G. pulchrum* rDNA (Makouloutou et al. 2013a) with a few divergences of nucleotide repeat units (Table 4).

Three long sequences, 6546 bp in length, from the 5′-terminus of 18S to 28S rDNA of *G. aegypti* from spiny mice were obtained. These sequences included 1814-bp-long partial 18S rDNA, 532-bp-long ITS1, 158-bp-long 5.8S rDNA, 463-bp-long ITS2, and 3579-bp-long partial 28S rDNA. Similarly, two long sequences, 6578 bp in length, from the 5′- terminus of 18S to 28S rDNA of *G. neoplasticum* from a black rat were obtained. These sequences comprised 1814-bp-long partial 18S rDNA, 540-bp-long ITS1, 158-bp-long 5.8S rDNA, 478-bp-long ITS2, and 3588-bp-long partial 28S rDNA. These two rodent *Gongylonema* spp. had unique rDNA nucleotide sequences, different from those of *G. pulchrum*; only the 18S and 5.8S rDNA sequences showed relatively high identities of 97.2–98.7 %, while the remaining sequences were less than 75 % identical. The 18S, 5.8S, and 28S rDNA sequences of the two rodent *Gongylonema* spp. showed no intraspecific variation, with nucleotide identities being 99.8 % (1811/1814), 100 % (158/158), and 98.9 % (3550/3590), respectively. More than a half of the nucleotide variations (24 out of 40 nucleotide sites) were found in the initial 900-bp-long

sequences of 28S rDNA of these two rodent *Gongylonema* spp. Both the ITS1 and ITS2 regions of *G. aegypti* and *G. neoplasticum* were highly diverse with 66.1–67.9 % nucleotide identities.

1. 3. 3 *cox-1* of *G. pulchrum* in Alashan League, *G. aegypti* and *G. neoplasticum*

A partial *cox-1* region amplified by the primer pair BpCoxI-F1 and BpCoxI-R1, 369-bp in length, of *G. pulchrum* mtDNA was successfully sequenced and compared with our previous data of the same species from deer, cattle, and squirrel monkeys in captivity (Makouloutou et al. 2013a) (Table 5). The *cox-1* nucleotide sequences of *G. pulchrum* from cattle in Inner Mongolia, China, were divided into two haplotypes. Two samples of one haplotype (worms #8 and #9) and eight samples of the other haplotype (worms #2, #3, #5–#7, and #10–#12) were further amplified by the primer pair StrCoxAfrF and MH28R, and longer *cox-1* sequences, 841-bp in total length, were successfully sequenced. In the 369-bp long *cox-1* sequences, these two haplotypes had five nucleotide substitutions at 589, 637, 688, 799, and 826 base sites. In the 841-bp sequences, 10 more nucleotide substitutions were observed at 16, 73, 115, 166, 175, 196, 259, 326, 424, and 454 base sites relative to the 5′-end of the *cox-1* sequence of *G. pulchrum* from Alashan League (DDBJ/EMBL/GenBank accession nos. LC026035, LC026036, and LC026038– LC026045). Despite these numerous nucleotide substitutions, the amino acid sequences (280-aa long) of the sequenced *cox-1* region were absolutely identical, due to almost all of the nucleotide substitutions occurring at the third base of each codon.

Three and one *cox-1* nucleotide sequences of *G. aegypti* and *G. neoplasticum* were successfully amplified, respectively. Comparable 369-bp lengths of the *cox-1* region of the former species showed three haplotypes, 98.9 % (365/369)–99.2 % (366/369) identities. Between these two rodent *Gongylonema* spp., the identities of *cox-1* nucleotide sequences were

91.6 % (338/369)–92.1 % (340/369); the amino acid sequences were absolutely identical. Between the *Gongylonema* spp. from cattle and rodents, nucleotide identities ranged between 89.2 % (329/369) and 90.2 % (333/369), with an amino acid identity of 95.9 % (118/123). The relationships of *cox-1* haplotypes of different *Gongylonema* spp. with different origins are illustrated in Fig. 4. The figure shows the following: (1) *G. pulchrum* from cattle in Inner Mongolia, China, were divided into two haplotypes like the gullet worms from cattle in Japan; (2) *G. pulchrum* haplotypes from cattle were distinct from the varied haplotypes of *G. pulchrum* from wild mammals such as deer, wild boars, and macaque monkeys in Japan; and (3) rodent *Gongylonema* spp., *G. aegypti* and *G. neoplasticum*, had their own haplotypes, clearly distinct from *G. pulchrum*. This is akin to the relationship between *G. pulchrum* and the gullet worm from water buffalo in Nepal, tentatively named as '*G*. cf. *pulchrum*', due to the paucity of critical morphological uniqueness and scant biogeographical data in our previous study (Makouloutou et al. 2013b). Consequently, the genetic data collected here provide support for the consideration of '*G*. cf. *pulchrum*' in water buffalo in Nepal as a new species, *Gongylonema nepalensis* n. sp.

1. 3. 4 Description

***G. nepalensis* n. sp. (Nematoda: Spirurida: Gongylonematidae)**

Morphologically, the present species is identical to *G. pulchrum* except for shorter left spicules. For morphometric characters, see Makouloutou et al. (2013b) and Table 3 in the present study.

Taxonomic summary

Host: *Bubalus bubalis* (Linnaeus, 1758), water buffalo (Artiodactyla: Bovidae).

Locality: Chitwan and Kathmandu, Nepal.

Site of infection: Esophageal mucosa.

Materials deposited: Holotype, NSMT-As3673a; allotype, NSMT-As3672a; paratypes, NSMT-As3673b, 3672b, 3674–3691.

Etymology: The species name comes from the country where worms were collected.

Prevalence: Five out of 58 (8.6 %) water buffaloes in Chitwan, and 13 out of 53 (24.5 %) water buffaloes in Kathmandu, Nepal.

1. 3. 5 Remarks

Morphologically, the present species is almost identical to *G. pulchrum* collected from cattle, except for the markedly shorter left spicule relative to its body size (Makouloutou et al. 2013b). Genetic analyses of its partial rDNA sequence showed 99.8, 100, and 98.3–98.8 % nucleotide identities with those of *G. pulchrum* collected from cattle, sika deer, wild boars, and captive squirrel monkeys in Japan (Makouloutou et al. 2013b). As shown in the present study, the rDNA sequences of two rodent *Gongylonema* spp., *G. aegypti* and *G. neoplasticum*, showed 99.8, 100, and 98.9 % nucleotide identities with each other. The ITS regions of *G. pulchrum* and *G. nepalensis* n. sp. showed 56–88 % nucleotide identities, whereas the two rodent *Gongylonema* spp. showed 66–68 % nucleotide identities. Between these two rodent *Gongylonema* spp., the identities of *cox-1* nucleotide sequences were 91.6 % (338/369)–92.1 % (340/369); the amino acid sequences were absolutely identical. The identities of *cox-1* nucleotide sequences of *G. pulchrum* of different origins and *G. nepalensis* n. sp. were 89.2 % (329/369)–90.2 % (333/369), with one amino acid substitution (Makouloutou et al. 2013b). Given the genetic relationship of *G. nepalensis* n. sp. to the other *Gongylonema* spp. hitherto

examined, it is appropriate to countenance '*G.* cf. *pulchrum*' from water buffalo documented by Makouloutou et al. (2013b) as an independent species from other *Gongylonema* spp. As stated in Makouloutou et al. (2013b), the host specificity and geographical distribution of *G. nepalensis* n. sp. remain to be clarified in future studies.

1. 4 Discussion

Data on the prevalence of gullet worms in China are limited. Human case reports have indicated more than 62 cases (Chen 1982) or 101 cases (Xu et al. 2000) in China, although *G. pulchrum* infection in Chinese people appears to be underestimated in European journals because most of the reports are written in Chinese or published in national journals in China. For example, Haruki et al. (2005) counted only six human cases in China out of 52 human records of gongylonemosis worldwide. Furthermore, Chen (1982) reported that Chinese patients often had infection with multiple worms; of 51 cases, there were 15 cases with two worms, 10 cases with three worms, 16 cases with four worms, and 1 each with five, eight, nine, and 16 worms. Zhu et al. (2012) recently reported an incidence of 31.5 % of *G. pulchrum* eggs in 76 fecal samples of wild Tibetan macaques (*Macaca thibetana*) in Mt. Huangshan, China. These circumstantial data from limited host species suggest a high prevalence of *G. pulchrum* in domestic and wild mammals in China. Thus, it is not unexpected to record *G. pulchrum* from 47.4 % of goats and 72.1 % of cattle in Alashan League, Inner Mongolia, China.

The gullet worms collected from cattle in Inner Mongolia showed the cattle genotype of ITS1 and ITS2, and the 12 isolates were divided into two *cox-1* haplotypes, closely related to two *cox-1* haplotypes of the worms from cattle in Japan (Makouloutou et al. 2013a). One haplotype was identical to *G. pulchrum* from cattle in Iran, captive squirrel monkeys in

zoological gardens in Japan, and Hokkaido sika deer, and closely related (single nucleotide substitution) to the 'cattle-I' haplotype of *G. pulchrum* in Japanese cattle (Fig. 4). Similarly, the other haplotype of the gullet worms from China was closely related (single nucleotide substitution) to the 'cattle-II' haplotype of *G. pulchrum* in Japanese cattle (Fig. 4). As mentioned above, we suspect the 'cattle-II' haplotype of *G. pulchrum* might be prevalent in Asian cattle, while the 'cattle-I' haplotype might spread worldwide through global distribution of European breeds of cattle or other domestic ruminants. The higher prevalence of the former haplotype of *G. pulchrum* in cattle from Inner Mongolia prompted us to elucidate different origins of the two haplotypes of the worms. However, as this is not conclusive, further molecular genetic surveys on *G. pulchrum* from cattle in Asian countries where no European cattle and sheep have been introduced or in the European continent free from Asian cattle are required.

In the present study, we provide for the first time the long rDNA sequences as well as partial *cox-1* sequences of *Gongylonema* worms other than *G. pulchrum*, enabling us to understand interspecific genetic divergences of *Gongylonema* spp. *G. aegypti* was originally described by Ashour and Lewis (1986) for spiruroid nematodes isolated from the gastric mucosa of five of 104 *Mus musculus* and one of 128 *Gerbillus gerbillus* from Abu-Rawash, Giza Province, Cairo, Egypt. *G. neoplasticum*, which has cockroaches (*Periplaneta americana*, *Periplaneta orientalis*, and *Blatta germanica*) as its intermediate host, was described from brown rats (*Rattus norvegicus*) imported probably from Estonia to Denmark as well as brown rats and black rats in a sugar refinery in Copenhagen (Fibiger and Ditlevsen 1914). Experimentally, Fibiger and Ditlevsen (1914) could infect mice (*M. musculus*), rabbits, and guinea pigs by feeding them with infected cockroaches. The genetic divergences of rDNA and

partial *cox-1* between these two rodent *Gongylonema* spp. are equal to those between *G. pulchrum* in cattle/wild mammals (e.g., sika deer, wild boars, and Japanese macaques) and '*G.* cf. *pulchrum*' in water buffalo documented by Makouloutou et al. (2013b). Although the previous study (Makouloutou et al. 2013b) could not ascertain the significance of the genetic distinctness of '*G.* cf. *pulchrum*' in water buffalo from *G. pulchrum* in other mammals due to a lack of information on genetic divergences between different *Gongylonema* spp., the genetic data collected here provide support for the consideration of '*G.* cf. *pulchrum*' in water buffalo in Nepal as a new species, *G. nepalensis* n. sp. Further accumulation of genetic data on diverse *Gongylonema* spp. with different host and geographical origins could be clearly useful to understand the relationships of various taxa of the genus.

Chapter II

Molecular genetic diversity of *Gongylonema neoplasticum* (Fibiger and Ditlevsen, 1914) (Spirurida: Gongylonematidae) from rodents in Southeast Asia

The work described in the chapter has been published as follows:

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Abstract

More than a dozen *Gongylonema* spp. (Spirurida: Spiruroidea: Gongylonematidae) have been described from a variety of rodent hosts worldwide. *Gongylonema neoplasticum* (Fibiger and Ditlevsen, 1914), which dwells in the gastric mucosa of rats such as *Rattus norvegicus* (Berkenhout) and *Rattus rattus* (Linnaeus), is currently regarded as a cosmopolitan nematode in accordance with global dispersion of its definitive hosts beyond Asia. To facilitate the reliable specific differentiation of local rodent *Gongylonema* spp. from the cosmopolitan congener, the genetic characterization of *G. neoplasticum* from Asian *Rattus* spp. in the original endemic area should be considered since the morphological identification of *Gongylonema* spp. is often difficult due to variations of critical phenotypical characters, e.g. spicule lengths and numbers of caudal papillae. In the present study, morphologically identified *G. neoplasticum* from 114 rats of seven species from Southeast Asia were selected from archived survey materials from almost 4500 rodents: Thailand (58 rats), Cambodia (52 rats), Laos (three rats) and Philippines (one rat). In addition, several specimens from four rats in Indonesia were used in the study. Nucleotide sequences of the ribosomal RNA gene (rDNA) (5649 bp) and the cytochrome *c* oxidase subunit 1 gene (*cox-1*) (818 bp) were characterized. The rDNA showed little nucleotide variation, including the internal transcribed spacer (ITS) regions. The *cox-1* showed 24 haplotypes, with up to 15 (1.83%) nucleotide substitutions regardless of parasite origin. Considering that *Rattus* spp. have been shown to originate from the southern region of Asia and *G. neoplasticum* is their endogenous parasite, it is reasonable to propose that the present study covers a wide spectrum of the genetic diversity of *G. neoplasticum*, useful for both the molecular genetic speculation of the species and the molecular genetic differentiation of other local rodent *Gongylonema* spp. from the cosmopolitan congener.

2. 1 Introduction

Members of the genus *Gongylonema* Molin, 1857 (Spirurida: Spiruroidea: Gongylonematidae), are filiform nematodes dwelling in the mucosa of the upper digestive tract of a variety of mammals and birds worldwide (Yamaguti 1961; Skrjabin et al. 1967; Lichtenfels 1971; Anderson 1992). The worms are characterized by verruciform thickenings, i.e. longitudinal rows of cuticular bosses, on the anterior surface of the body (Chabaud 2009). More than a dozen nominal *Gongylonema* spp. have been described from rodents worldwide based on morphological criteria (Fibiger & Ditlevsen 1914; Kruidenier and Peebles 1958; Yamaguti 1961; Skrjabin et al. 1967; Gupta and Trivedi 1985; Ashour and Lewis 1986; Diouf et al. 1997; Kinsella et al. 2016). Some of the described species require the collection and characterization of more specimens as their characterization was based on a limited number of worms or they were recovered from a unique body location, different from other species, as indicated by Kinsella et al. (2016).

Considering an earlier trend where many helminth species descriptions were primarily based on different isolation sources and/or some morphological uniqueness of microscopically observed worms, it would be prudent to discern the taxonomic relationships of local *Gongylonema* spp. isolated from different rodent hosts in the world, as has been done for *G. pulchrum* Molin, 1857 with many synonymized taxa based on cross infection experiments (Ransom and Hall, 1915; Baylis et al. 1926a, b; Schwartz and Lucker 1931; Lucker 1932) or meticulous morphological analyses (Schwartz and Lucker 1931; Lichtenfels 1971). These strategies for taxonomical revision can be hampered by the practical difficulties of worm collection from wild rodent hosts and/or collection of wild rodents for experimental infection

purposes; however, molecular genetic analyses now offer an alternative approach for such a task.

Nucleotide sequencing of the ribosomal RNA gene (rDNA) and partial cytochrome *c* oxidase subunit 1 (*cox-1*) region of mitochondrial DNA (mtDNA) of specimens of *Gongylonema* isolated from different mammalian hosts has enabled us to differentiate *G. nepalensis* Setsuda, Da, Hasegawa, Behnke, Rana and Sato, 2016 from *G. pulchrum* and understand their possible natural transmission dynamics in domestic and wild ruminants (Sato 2009; Makouloutou et al. 2013a, b; Varcasia et al. 2017; Chapter I of the present study). We recently genetically characterised for the first time two rodent *Gongylonema* spp., i.e. *G. neoplasticum* from the black rat (*Rattus rattus* (Linnaeus)) on Okinawa Island, Japan, and *G. aegypti* Ashour and Lewis, 1986 from the Arabian spiny mouse *Acomys dimidiatus* (Cretzschmar, 1826) on the Sinai Peninsula, Egypt, disclosing their distinctness but close relatedness (Chapter I). Considering that *Rattus norvegicus* (Berkenhout) (brown rats) and *R. rattus*, the dominant hosts for *G. neoplasticum* worldwide (Wells et al. 2015), originated from southern China and Southeast or South Asia (Aplin et al. 2011; Song et al. 2014; Thomson et al. 2014; Puckett et al. 2016), the greatest genetic diversity of their endogenous parasites would be expected to be found in worms collected in Southeast Asia rather than invaded localities beyond South and Southeast Asia (Morand et al. 2015), such as Japan, the sole locality of available molecular data for *G. neoplasticum*. In the latter case, worms must have survived in their new environment by way of the bottleneck phenomenon, thus leading to lower genetic diversity.

In the present study, specimens of *Gongylonema* in the stomach of *Rattus* spp. (*R. norvegicus*, *R. exulans* (Peale), *R. tanezumi* (Temminck), *R. andamanensis* Hinton, and another

Rattus sp.), *Maxomys surifer* (Miller), and *Berylmys bowersi* (Anderson) collected in Cambodia, Indonesia, Laos, Philippines and Thailand were examined for their genetic diversity in their putative native areas.

2. 2 Materials and methods

2. 2. 1 Collection of parasites and morphological observation

During the last 10 years, a variety of murine rodents (approximately 4500 individuals of more than 20 species) has been trapped in Cambodia, Laos, Philippines and Thailand to try and understand the role of host species and habitat on helminth species richness and to also answer other ecological and epidemiological questions related to parasitic diseases (e.g. Pakdeenarong et al. 2014; Palmeirim et al. 2014; Chaisiri et al. 2015, 2016; Veciana et al. 2015; Ribas et al. 2016). As part of these studies, specimens of *Gongylonema* were recorded from various murine hosts (Pakdeenarong et al. 2014; Palmeirim et al. 2014; Chaisiri et al. 2016; Ribas et al. 2016), a portion of which was used for the present study; 114 worms collected from different individuals of five *Rattus* spp., *M. surifer*, and *B. bowersi* trapped in Thailand (11 localities), Cambodia (three localities), Lao PDR (three localities), and Philippines (one locality) during the period February 2008 to August 2014 (Table 6). To increase sampling areas, 13 worms from four brown rats trapped in a wet market in Surabaya city, Indonesia, in September 2017 were included in the present study (Table 6). Individual worms embedded in the gastric mucosa were carefully removed from the tissue using fine forceps and fixed individually in 70% ethanol.

Nine of the 114 worms chosen from archived survey materials were male. Six male and six female worms displaying no morphological damage were selected for morphological observation. Similarly, six male and three female worms collected in Indonesia were used for

morphological examination. Specimens preserved in 70% ethanol were placed in a clearing solution with glycerol and lactic acid, and observed under a light microscope. Figures were drawn with the aid of a camera lucida. Measurements were performed on these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) when necessary.

2. 2. 2 DNA extraction, polymerase chain reaction (PCR), and sequencing

The middle 1/5–1/3 section of 109 female worms and 2.5-mm long segments of two male worms were individually used for DNA extraction. Each sample was washed three times in distilled water, placed in a clean 1.5-ml plastic tube, freeze-dried (freeze dryer model EYELA FD-5N; Tokyo Rikakikai Co., Bunkyo-ku, Tokyo, Japan), then crushed with an individual clean plastic pestle. Parasite DNAs were extracted separately from these samples using an IllustraTM tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions.

PCR amplification of overlapping rDNA fragments was performed in a 20µl volume containing a DNA polymerase, Blend Taq-Plus- (TOYOBO, Dojima Hama, Osaka, Japan), and universal eukaryotic primer pairs as previously described (Makouloutou et al., 2013a). PCR products for sequencing were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan). Following direct sequencing of PCR amplicons, sequences were assembled manually with the aid of the CLUSTAL W multiple alignment program (Thompson et al. 1994). For rDNA segments containing the internal transcribed spacer (ITS) regions, the amplicon was cloned into a plasmid vector, pTA2 (TArget CloneTM; TOYOBO), and transformed into Escherichia coli JM109 cells (TOYOBO) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid

Mini Kit (NIPPON Genetics Co.) and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

The *cox-1* region of mtDNA was amplified by two different primer pairs as follows: (i) Gpul_Cox1-303F (5'-GGC TCC TGA GAT GGC TTT TC-3') and Gpul_Cox1-R (5'-ATG AAA ATG TGC CAC TAC ATA ATA TGT ATC-3'); and (ii) Gpul_Cox1-403F (5'-CCT GGT GGT AGC TGA ACT TT-3') and Gpul_Cox1-906R (5'-GCC CCA AAC AGA CGT ACC TA-3'). These primers were designed using online software 'Primer3web ver.4.0.0' (Untergasser et al. 2012) and referring to a complete *cox-1* nucleotide sequence of *G. pulchrum* (DDBJ/ EMBL/GenBank: KM264298; Liu et al. 2015). PCRs were conducted in a thermal cycler using the following cycling protocol: 3 min at 94˚C, followed by 40 cycles at 94˚C for 45 s, 48˚C for 1 min, and 72˚C for 1 min, then a final extension at 72˚C for 7 min. For Indonesian worms, another primer pair, Gpul_Cox1-F (5' -GTG GTT TTG GTA ATT GAA TGC TA-3') and Gpul_Cox1-R, was used to amplify *cox-1* nucleotide sequences, according to Varcasia et al. (2017). Amplicons were sequenced after purification as described above. For sequencing of 868 bp or 905 bp long *cox-1* products, which included 50 bp or 53 bp long primer-annealing areas, respectively, the five PCR amplification primers detailed above were used.

The nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank databases under the accession numbers LC331001–LC331051 and LC334451–LC334454. Voucher specimens for these DNA analyses were deposited in the National Museum of Nature and Science, Tokyo, Japan, under the accession numbers As4306–As4423.

2. 2. 3 Phylogenetic analysis

For phylogenetic analysis, the newly obtained *cox-1* sequences (818 bp in length) of *Gongylonema* worms examined in the present study and those of the same genus retrieved from the DDBJ/EMBL/GenBank databases were used. *Spirocerca lupi* (Rudolphi, 1809) (Spirurida: Thelaziidae; GenBank: KC305876), *Dirofilaria repens* Railliet and Henry, 1911 (Spirurida: Onchocercidae; GenBank: KX265048), and *Onchocerca volvulus* (Leuckart, 1893) (Spirurida: Onchocercidae; GenBank: AP017695) were retrieved from the databases and used as an outgroup for the construction of the phylogenetic tree. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the 'phylogeny.fr' website (http://www.phylogeny.fr/) using 818 characters, of which 258 were variable. The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

2. 2. 4 *cox-1* haplotype analysis

The relationships of different haplotypes based on 369 bp long *cox-1* nucleotide sequences were visualized using an automated haplotype network layout and visualisation software, HapStar, downloaded at http://fo.am/hapstar (Teacher and Griffiths 2011).

2. 3 Results

2. 3. 1 Morphology of *G. neoplasticum* from Asian rats

The number of worms embedded in the gastric mucosa of each rat selected for this study from archived survey materials (a total of 114 rats of seven different species trapped at 18 localities in four countries) ranged from a few to several; a single worm from each rat was used

(Table 6). In addition, nine worms from two brown rats trapped in Surabaya city, Indonesia, were used for morphological observation. Worms showed marked sexual dimorphism, evident in worm sizes (distinctly smaller sizes of male worms; see Table 7) and differently developed cuticular bosses in the anterior part of the body (poor in male worms and well developed in female worms; see Fig. 5). Mouth opening was connected to the short pharynx, then followed by the muscular and glandular esophagi, and intestine. Male worms with asymmetric caudal alae had eight pairs of caudal papillae (four precloacal and four post-cloacal), in addition to a pair of phasmids near the posterior extremity. One of six male worms had an additional caudal papilla which was located at the anteriormost position of the pre-cloacal papillae on the left side (Fig. 5e). Male worms possessed a long left and a short right spicule (Fig. 5e). Left spicules were fine thread-like with round distal ends, whereas right spicules and gubernacular were squat. Measurements of the collected worms were well coincident with those of *G. neoplasticum* recorded in earlier studies (Table 7).

2. 3. 2 rDNA of *G. neoplasticum* from Asian rats of different origins

Following a preliminary reactivity check of rDNA segment amplification by PCR, the rDNA nucleotide sequences of several arbitrarily chosen worms were sequenced (Table 8); 5649 bp in length from near the 5'-terminus of 18S to 28S rDNA was comprised of 1814 bp long partial 18S rDNA, 540 bp long ITS1, 158 bp long 5.8S rDNA, 478 bp long ITS2, and 2659 bp long partial 28S rDNA. The nucleotide sequences of different worms were almost completely identical to one another, as well as to male and female worms of *G. neoplasticum* from the black rat in Okinawa, Japan (DDBJ/EMBL/GenBank: LC026032 and LC026033;

Chapter I). The few nucleotide substitutions observed were located at positions 437, 579, 814 and 1019 of the 28S rDNA (Table 9).

2. 3. 3 *cox-1* of *G. neoplasticum* from Asian rats of different origins

A partial *cox-1* region, 818 bp or 852 bp in length, was successfully sequenced in 55 of the collected worms (Table 10), showing 24 haplotypes with mostly only a few nucleotide substitutions and a maximum of 15 (1.83%) nucleotide substitutions. The most prominent haplotypes with one or no nucleotide substitution were found in 27 worms (49.1%) of different localities and host origins. In an ML phylogenetic tree constructed on the basis of these 818 bp long *cox-1* sequences, all specimens of *G. neoplasticum* from Asian rats formed a well-supported clade, which was distinct from *G. aegypti* from the Arabian spiny mouse in Egypt, a clade of *G. pulchrum* from domestic ruminants in Japan and China, and *G. nepalensis* from ruminants on Sardinia Island, Italy (Fig. 6). To define the molecular genetic relationship with a specimen of *G. neoplasticum* from the black rat in Okinawa, Japan (DDBJ/EMBL/GenBank: LC026049; Chapter I), 369 bp long *cox-1* segments (constituting the 450th nucleotide through to the 3'-terminus of the 818 bp long *cox-1* fragments) of the 55 successfully sequenced worms were analyzed by the HapStar network illustration (Fig. 7). These 369 bp long *cox-1* segments contained the majority of nucleotide substitutions (92 sites), whereas the anterior 449 bp long segments contained only 24 nucleotide substitution sites, when specimens of *G. pulchrum*, *G. nepalensis*, *G. aegypti*, *G. neoplasticum* and *Gongylonema* collected in the present study were compared. When the 55 specimens *Gongylonema* collected in the present study were compared, the anterior 449 bp long *cox-1* segment contained 16 nucleotide substitution sites, and the posterior 369 bp long *cox-1* segment contained 20 nucleotide substitution sites. Subsequent

analyses with the 369 bp long *cox-1* segments showed 19 haplotypes; the most prominent haplotype was found in 30 worms (54.6%). Translation of amino acid (aa) sequences from the 818 bp and 369 bp long *cox-1* nucleotide sequences resulted in 17 types of 272 aa sequences and 10 types of 123 aa sequences, respectively. The most prominent amino acid sequence type in each analysis was found in 56.4% (31/55; 272 aa sequences) and 83.6% (46/55; 123 aa sequences) of analyzed worms. The *cox-1* haplotype of *G. neoplasticum* collected in Okinawa, Japan, was identical to the most prominent haplotype of the *Gongylonema* worms collected in Southeast Asian countries (Fig. 7), and its amino acid sequence, as well as that of *G. aegypti*, was identical to the most prominent amino acid sequence type in worms collected in the present study.

2. 4 Discussion

The *Gongylonema* worms collected in the present study appear to be a single species, *G. neoplasticum*, based on morphological characters such as continuous lateral alae, numbers of caudal papillae (four pairs of pre- and four pairs of post-cloacal ones), poor development of cuticular bosses on the anterior surface of male worms in contrast to developed ones in female worms (Fig. 5), in addition to specimen measurements (Table 7). Natural definitive hosts of the species include not only *R. norvegicus* and *R. rattus*, but also *Bunomys chrysocomus* (Hoffmann) (yellow-haired hill rat), *Bandicota savilei* Thomas (Savile's bandicoot rat), *Maxomys surifer* (red spiny rat), *Mus caroli* (Ryukyu mouse), *Mus cervicolor* Hodgson (fawn-colored mouse), *Mus cookii* Ryley (Cook's mouse), *Niviventer fulvescens* (Gray) (chestnut white-bellied rat), *Rattus exulans* (Polynesian rat), *Rattus losea* (Swinhoe) (lesser ricefield rat), *Rattus tanezumi* (Asian house rat), *Rattus tiomanicus* (Miller) (Malayan field rat) and

Oryctolagus cuniculus (Linnaeus) (European rabbit) (Fibiger and Ditlevsen 1914; Yokogawa 1925; Kruidenier and Peebles 1958; Skrjabin et al. 1967; Singh and Cheong 1971; Yap et al. 1977; Leong et al. 1979; Krishnasamy et al. 1980; Jueco and Zabala 1990; Hasegawa and Syafruddin 1995; Eira et al. 2006; Syed-Arnex and Mohd Zain 2006; Paramasvaran et al. 2009; Dewi 2011; Chaisiri et al. 2012; Paramasvaran et al. 2012; Dewi and Purwaningsih 2013). As detailed measurements of specimens from different hosts or localities have not always been recorded, possible variations of phenotypical characters of *G. neoplasticum* have not been assessed to any great extent. Without any knowledge of the genetic background of worms under investigation, i.e. worms of a single species or multiple species, it is impossible to explain the significance of possible phenotypical variations. Due to this reason, Kinsella et al. (2016) stressed the importance of acquiring molecular data in addition to phenotypical character data from collected parasites to understand the systematics of rodent *Gongylonema* spp. The present study aimed to characterize the rDNA and *cox-1* nucleotide sequences of *G. neoplasticum* based on material collected as part of several helminth surveys conducted in Thailand, Cambodia, Laos and Philippines during the period February 2008 to August 2014, with additional worms from Indonesia (Pakdeenarong et al. 2014; Palmeirim et al. 2014; Chaisiri et al. 2015, 2016; Veciana et al. 2015; Ribas et al. 2016). The majority of worms collected in these surveys had previously undergone microscopic observation for their specific identification. Furthermore, a portion of the worms had been preserved for several years, dating from February 2008 through to the spring of 2016. Therefore, at the outset of our study, we were aware that these past treatments of the samples could negatively affect the PCR amplification of rDNA and *cox-1* mtDNA fragments. Indeed, successful *cox-1* sequencing was achieved in 47.7% (51/107) of examined worms from archived survey materials.

Using several arbitrarily chosen worms, almost identical rDNA sequences (including the ITS regions) with only a few nucleotide substitutions over a length of 5649 bp were obtained. The ITS regions are highly variable nuclear DNA regions useful for species and strain separation. In the case of *G. pulchrum*, numerous repeats of a few to several nucleotide units often occur in the ITS regions, and intraspecific as well as intra-individual variations of these nucleotide repeats have been seen in addition to interspecific variations (Makouloutou et al., 2013a; Chapter I). Similarly, *G. neoplasticum* collected from Southeast Asian rats exhibited such nucleotide repeats in the ITS regions, but lacked variation in the number of repeats of certain nucleotide units. The rDNA nucleotide sequences of *G. neoplasticum* worms collected in the present study were almost completely identical (only a few nucleotides differed) to those of *G. neoplasticum* isolated in Okinawa, Japan (DDBJ/EMBL/GenBank: LC026032 and LC026033). Therefore, as the unique rDNA sequences of *G. neoplasticum* and those of congeners such as *G. aegypti*, *G. pulchrum* and *G. nepalensis* were discussed in Chapter I, we do not repeat that discussion here.

Makouloutou et al. (2013a) reported a great variety of *cox-1* nucleotide sequences (seven *cox-1* haplotypes), but only a small amount of amino acid sequence variation, in *G. pulchrum* isolated from wild mammals such as deer, wild boars and Japanese macaques in Japan. This is in contrast to only two major *cox-1* haplotypes in cattle in Japan, China (Inner Mongolia) and Iran (Halajian et al. 2010; Makouloutou et al. 2013a; Chapter I). This might reflect the fact that endemic mammals have a parasite population with a spectrum of genetic diversity, whereas mammals translocated by human activities have a parasite population with little genetic diversity. Considering that *G. neoplasticum* is currently cosmopolitan in distribution with an unintended introduction of its rodent hosts as a consequence of recent global trade, and that

Rattus spp. such as *R. norvegicus*, *R. rattus*, *R. tanezumi*, and *R. exulans* have been shown to originate in southern China and Southeast or South Asia (Aplin et al. 2011; Song et al. 2014; Thomson et al. 2014; Puckett et al. 2016), it is reasonable to propose that *G. neoplasticum* examined here is likely to have a maximum spectrum of genetic diversity in fast-evolving mtDNA genes such as *cox-1*.

As hypothesised above, the *cox-1* nucleotide sequences of *G. neoplasticum* examined in the present study showed a high genetic diversity, represented by the presence of 24 haplotypes (based on 818 bp long sequences) or 19 haplotypes (based on 369 bp long sequences) regardless of collection site (country) and host rat species (Fig. 7). When these 818 bp and 369 bp long nucleotide sequences were translated to amino acid sequences, 17 and 10 types of sequences were differentiated, with the most prominent sequence found in 56.4% (31/55) and 83.6% (46/55) of analyzed worms, respectively. This finding indicates that most of the *cox-1* nucleotide substitutions of samples of *G. neoplasticum* examined in the present study occurred at the third nucleotide of codons, as previously observed in an earlier study described in Chapter I. As far as examined here, similar to *G. pulchrum* isolated from wild mammals in Japan, there is no suggestion of colonisation of special haplotypes of *G. neoplasticum* at defined localities nor prevalence of special haplotypes in defined rat species. Since known intermediate hosts (e.g. common insects such as cockroaches and beetles (Fibiger and Ditlevsen 1914; Yokogawa 1925; Dittrich 1963) and definitive hosts (different rat species) for *G. neoplasticum* are sympatric and probably have comparable susceptibilities to infection with this spirurid nematode, the current wide distribution of genetically heterogeneous *G. neoplasticum* with different *cox-1* haplotypes in Southeast Asia could be a natural outcome. On the contrary, the lower genetic heterogeneity of *G. neoplasticum* in localities where black and brown rats were introduced as a consequence

of recent global trade is highly predictable in view of the bottleneck phenomenon (Morand et al., 2015).

A possible genetic spectrum of *G. neoplasticum* from rats distributed in their original endemic area, Southeast Asia, is of great importance, particularly when only a single (or a few) *Gongylonema* worm from a rodent host at a certain locality is collected and analyzed for its genetic uniqueness. As mentioned earlier, more than a dozen rodent *Gongylonema* spp. have been recorded to date. The molecular characterization of each species should facilitate the phenotypical characterization which often shows variation. Such efforts may detect substantial specific diversities of rodent *Gongylonema* spp., as previously communicated by Kinsella et al. (2016).

Chapter III

Gongylonema infection of wild mammals in Japan and Sardinia (Italy)

The work described in the chapter has been published as follows:

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Abstract

The gullet worms, classical *Gongylonema pulchrum* and newly differentiated *Gongylonema nepalensis*, are prevalent in various mammals in Japan and Sardinia Island, Italy, respectively. The former species is cosmopolitan in distribution, dwelling in the mucosa of the upper digestive tract of a variety of domestic and wild mammals and also humans. At present, geographical distribution of *G. nepalensis* is known in Nepal and Sardinia, with the nematode having been recorded from the oesophagus of water buffaloes (Nepal), cattle, sheep, goats, and wild mouflon (Sardinia). To clarify their natural transmission cycles among domestic and wild mammals, the present study analyzed the ribosomal RNA gene (rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox-1*) of worms with various origins; *G. pulchrum* worms from sika deer, wild boars, Japanese macaques, and feral alien Reeves's muntjacs (*Muntiacus reevesi*) in Japan, and *G. nepalensis* worms from a red fox and a wild boar in Sardinia. Although the internal transcribed spacer (ITS) regions of rDNA and partial *cox-1* nucleotide sequences of *G. pulchrum* from native wild mammals in Japan were distinct from those of the worms in cattle, the worms from feral alien Reeves's muntjacs showed the cattle-type ITS genotype and *cox-1* cattle-I and II haplotypes. The rDNA and *cox-1* nucleotide sequences of *G. nepalensis* from a red fox in Sardinia were almost identical to those of the worms from domestic and wild ruminants on the island. The ecological interaction between domestic and wild mammals and their susceptibility to different *Gongylonema* spp. must be considered when trying to elucidate this spirurid's transmission dynamics in nature.

3. 1 Introduction

Adult worms of the genus *Gongylonema* (Nematoda: Spirurida: Gongylonematidae) are easily recognized due to the verruciform cuticular thickenings of the anterior part of their bodies (Anderson 1992; Chabaud 2009). *Gongylonema pulchrum* (Molin, 1857), also known as the "gullet worm" because of its localization in the epithelium of the upper digestive tract, is distributed worldwide and its definitive hosts are a variety of domestic and wild mammals including cattle, sheep, goats, donkeys, cervids, equines, camels, bears, pigs, non-human primates, and humans (Alicata 1935; Yamaguti 1961; Skrjabin et al. 1967; Zinter and Migaki 1970; Lichtenfels 1971; Kirkpatrick et al. 1986; Anderson 1992; Duncan et al. 1995; Brack 1996; Xu et al. 2000; Haruki et al. 2005; Sato et al. 2005; Chapter I). Transmission of the gullet worm to definitive hosts occurs through ingestion of intermediate hosts such as infected dung beetles or drinking water contaminated with third-stage larvae (Cappucci et al. 1982; Anderson 1992; Kudo et al. 1996).

With an almost identical morphology except for distinctly shorter left spicules relative to the entire body, *Gongylonema nepalensis* Setsuda et al., 2016, has recently been separated from *G. pulchrum* (Makouloutou et al. 2013b; Varcasia et al. 2017; Chapter I). This taxonomic revision was supported by molecular genetic analyses of the nuclear ribosomal RNA gene (rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox-1*) of *Gongylonema* worms of different origins (Chapter I). This species has been isolated from water buffaloes (*Bubalus bubalis*) in Nepal and cattle (*Bos taurus*), sheep (*Ovis aries*), goats (*Capra hircus*), and wild European mouflon (*Ovis aries musimon*) on the island of Sardinia, Italy (Makouloutou et al. 2013b; Varcasia et al. 2017).

As shown by our previous studies (Makouloutou et al. 2013a, b; Varcasia et al. 2017; Chapters I and II), molecular genetic analyses of the rDNA and *cox-1* sequences of *Gongylonema* worms allow us to speculate on the geographical distribution of different genetic lineages of the species and the transmission dynamics of worms among different host animals. In Japan, molecular approaches have proven that the transmission cycles of *G. pulchrum* in cattle and wild mammals such as sika deer (*Cervus nippon*), wild boars (*Sus scrofa leucomystax*), and Japanese macaques (*Macaca fuscata*) are distinct. Furthermore, they have led to the recognition of two genotypes (cattle-type and deer-type) of the rDNA, particularly the internal transcribed spacer (ITS) regions, and two major haplotypes (cattle-haplotype and wildlife-haplotype) of *cox-1*.

In the present study, we collected additional *Gongylonema* worms in Japan and Sardinia, including *G. pulchrum* from feral alien Reeves's muntjacs (*Muntiacus reevesi*) on Izu-Oshima Island, Tokyo, Japan, and *G. nepalensis* from a red fox (*Vulpes vulpes*) and a wild boar (*Sus scrofa meridionalis*) in Sardinia, and analyzed their genetic backgrounds to clarify the relationships with *G. pulchrum* and *G. nepalensis* populations prevalent in domestic and wild mammals in each country.

3.2 Materials and methods

3. 2. 1 Parasite collection and morphological examination

Full-length esophagi of feral alien Reeves's muntjacs were collected on 26 January 2015 (seven animals), 25 and 26 July 2015 (15 animals), and 29 October 2016 (10 animals). Originally, a dozen captive Reeves's muntjacs escaped from Tokyo Municipal Oshima Park Zoo during a typhoon in the autumn of 1970 and subsequently became naturalized on the 91.1-

km^2 island. Control measures for this alien mammal species were initiated in 2007 and the total numbers of individuals trapped in 2014, 2015, and 2016 were 1022, 1412, and 2191, respectively (Tokyo Municipal Office 2017). The latest estimated number of alien Reeves's muntjacs on Izu-Oshima Island is *c*. 13,000. Permission for the academic use of viscera from trapped individuals was granted by the Tokyo Metropolitan Government Office.

All Reeves's muntjacs were matured adults with body weights of 6.6–10.0 kg. Their ages and sex were unknown. Frozen viscera including esophagi were transported to the Laboratory of Veterinary Parasitology, Yamaguchi University. The mucosal surface was checked carefully with the naked eye and individual worms were removed from the esophageal epithelium using fine forceps, and fixed in 70% ethanol or 10% neutral-buffered formalin solution.

Gongylonema worms were collected from the oral mucosa of a red fox on 31 January 2017 (shot in Urzulei, Ogliastra), from the esophageal mucosa of a wild boar on 15 February 2017 (slaughtered in Tortolì, Ogliastra), and from the esophageal mucosa of a domestic goat on 20 February 2017, at the Instituto Zooprofilattico Sperimentale della Sardegna, Tortolì, Ogliastra Province, Sardinia. Parasite samples were preserved in 70% ethanol.

Available male and female worms displaying no morphological damage were selected for morphological observation. Specimens preserved in 70% ethanol were placed on glasses to measure the body length and width, and cut the middle third of the entire body length for DNA extraction. The remaining anterior and posterior thirds of specimens were placed in a clearing solution with glycerol and lactic acid, and observed under a light microscope. Figures were drawn these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) when necessary. In addition, either the anterior or posterior part of the body length (less than half) of damaged specimens was used for DNA extraction. Collected specimens, excluding the portions

used for DNA extraction, were deposited in the National Museum of Nature and Science, Tokyo, Japan, under specimen numbers NSMT-As4426–4449.

3. 2. 2 DNA extraction, polymerase chain reaction (PCR), and sequencing

Parasite DNA was individually extracted from sections of worms (four worms from different Reeves's muntjacs, two from a goat, and one from a red fox) as described in Chapter I.

Polymerase chain reaction (PCR) amplification of partially overlapping rDNA fragments was performed in a 25-μl volume using different primer combinations as previously described (Makouloutou et al. 2013a). The *cox-1* region of *G. pulchrum* mitochondrial DNA (mtDNA) was amplified by a combination of two primers, Gpul_Cox1-F (5'-GTGGTTTTGGTAATTGAATGCTA-3') and Gpul_Cox1-R (5'-ATGAAAATGTGCCACTACATAATATGTATC-3'), as described by Varcasia et al. (2017). PCR amplification of *cox-1* gene was also conducted using 19 stock DNA samples of *G. pulchrum* from wild mammals stored at –20˚C (10 worms from four sika deer, five from five wild boars, and four from three Japanese macaques).

The purification of PCR products using a commercial kit and subsequent nucleotide sequencing, including that of rDNA genes containing ITS regions, were conducted as described in Chapter I. The obtained sequences were assembled with the aid of the CLUSTAL W multiple alignment program (Thompson et al. 1994).

New nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank databases under the accession numbers LC388743–LC388756 (rDNA) and LC388892–LC388914 (*cox-1*).

3. 2. 3 Phylogenetic analysis

For phylogenetic analysis, the newly obtained *cox-1* sequences, 852 bp in length, of *Gongylonema* worms collected in the present study and those of the same genus retrieved from the DDBJ/EMBL/GenBank databases were used. The accession numbers of the sequences analyzed in the present study are given in the figure showing the phylogenetic tree. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

As indicated in the previous reports (Makouloutou et al., 2013a, b; Chapter I), any parts of the rDNA, including ITS regions, show minimal intraspecific variation, and therefore phylogenetic analysis has not been conducted.

3. 3 Results

3. 3. 1 Morphological examination by light microscopy

One female worm was isolated from one of seven feral alien Reeves's muntjacs collected in January 2015, two male and five female worms from six of 15 animals collected in July 2015, and three male and four female worms from four of 10 animals collected in October 2016. Only two of the 11 parasitized Reeves's muntjacs were infected with more than one worm (either two or four worms). *Gongylonema* worm tracts only (i.e. devoid of worms) were detected in the esophageal mucosa of one Reeves's muntjac examined in January 2015 and one examined in October 2016 (Fig. 8). Worms were long and slender, embedding themselves in a zig-zag

manner in the esophageal mucosa. The anterior portion of male and female worms was characterized by prominent cuticular bosses with symmetrical lateral alae on both sides. The posterior portion of male worms was characterized by asymmetrical caudal alae with up to six pairs of papillae each in pre- and post-cloacal areas. The caudal end of female worms was bluntly conical and the vulva was situated relatively close to the posterior end. The eggs in female worms of unisexual infection were not fertilized. As shown in Table 11, the measurements of worms were comparable to those of *G. pulchrum* isolated from the esophageal mucosa of cattle, distinct from those of worms isolated from sika deer.

Two *Gongylonema* worms collected from the oral mucosa of a red fox were female (Fig. 9). One male worm and one female worm were collected from the esophageal mucosa of a wild boar. As shown in Table 11, these four worms were smaller than those of worms previously isolated from wild ruminants (European mouflon) and domestic animals in Sardinia. The manner of parasitism and external morphological features resembled those of *G. pulchrum* described above. However, the proportion of left spicule length to entire body length was 21.2%, comparable to that of *G. nepalensis* but distinctly smaller than that of *G. pulchrum.*

3. 3. 2 Molecular genetic analyses of the rDNA

Long nucleotide sequences of the rDNA of four *G. pulchrum* worms from feral alien Reeves's muntjacs were obtained. The nucleotide sequences of the 18S rDNA (1782 bp), 5.8S rDNA (158 bp), and 28S rDNA (3544 bp) were identical among the four worms. These sequences were also identical to those of several *G. pulchrum* isolates from cattle in Japan and China (e.g. DDBJ/EMBL/GenBank accession nos. AB513707, AB513711, and LC026017). Even with the remaining isolates from cattle, only one nucleotide substitution was detected in

the 18S rDNA and also in the 28S rDNA, whereas no substitutions were found in the 5.8S rDNA. Concerning the ITS1 (385–392 bp) and ITS2 (229 bp) regions, the nucleotide sequences of which show intra-individual and inter-individual variations (Chapter I), the ITS2 nucleotide sequences of the *G. pulchrum* isolates from Reeves's muntjacs had cattle-type repeats of units of a few nucleotide, which was distinct from the deer-type ITS2 nucleotide sequences (Chapter I).

Newly sequenced Sardinian *Gongylonema* isolates from a goat (two worms) and a red fox (one worm) showed virtually the same rDNA sequence as that of *G. nepalensis* from domestic and wild ruminants on the island (accession no. LC278392); one or two nucleotide substitutions in the 18S rDNA (minimum identity of 99.89% over 1782-bp length), no substitutions in the 5.8S rDNA (100% identity over 158-bp length), and one or two nucleotide substitutions in the 28S rDNA (minimum identity of 99.94% over 3535-bp length). These Sardinian *G. nepalensis* isolates from a goat and a red fox also showed intra-individual and inter-individual nucleotide variations in the ITS regions, with different numbers of repeats of a certain nucleotide (such as 'A') or two-nucleotide unit ('CA'). Table 12 shows the observed nucleotide variations in the ITS1 and ITS2 regions of Sardinian *G. nepalensis* isolates, spanning 397–412 bp and 237 or 240 bp, respectively.

3. 3. 3 Molecular genetic analyses of *cox-1*

Partial 852-bp long *cox-1* sequences of *G. pulchrum* isolates from Reeves's muntjacs (four worms) and native wild mammals such as sika deer, wild boars, and Japanese macaques (19 worms) in Japan, and Sardinian *G. nepalensis* from a goat and a red fox (three worms) were newly obtained. Nucleotide substitutions across the available 852-bp long *cox-1* sequences of

G. pulchrum and *G. nepalensis* occurred at 107 nucleotide positions (12.56% of all nucleotides), and interspecific differences were detected at 66 nucleotide positions. Two major haplotypes of *G. pulchrum* in cattle were based on 14 nucleotide substitutions (1.64% of all nucleotides). Three *G. pulchrum* worms from Reeves's muntjacs showed cattle-I haplotype, while one worm showed cattle-II haplotype; complete identities were observed to those in cattle (Chapter I). Although only a few nucleotide substitutions were seen among *G. pulchrum* worms of the same cattle haplotype, substitutions among *G. pulchrum* worms from wild mammals were found at 18 nucleotide positions over the same *cox-1* fragment length (accession nos. LC388896–LC388914).

As previously reported (Varcasia et al. 2017), all *cox-1* sequences of *G. nepalensis* from cattle, sheep, goats, and mouflon (accesssion no. LC278393), excluding one worm from a sheep (LC278394), showed absolute homology. One worm from a red fox examined in the present study (LC388893) showed one nucleotide substitution over 852 bp. This substitution was at a different nucleotide site from the aforementioned sheep worm (LC278394).

The translated amino acid (aa) sequences of *G. pulchrum* and *G. nepalensis* from a variety of mammals (analyzed sequences are shown in Fig. 10) based on the 852-bp long nucleotide sequences, i.e. 284 aa, were highly similar. The aa substitutions occurred at six sites (2.11%), indicating that most of the nucleotide substitutions of the *cox-1* fragments occurred at the third nucleotide position of codons, i.e. a substantial 97 out of 107 (90.65%) nucleotide substitution sites. The observed aa substitutions were not related to any specific group of *G. pulchrum* and *G. nepalensis*.

The phylogenetic relationships of the different isolates of *Gongylonema* spp. based on long *cox-1* nucleotide sequences are shown in Fig. 10. As typified by *G. pulchrum* worms in wild

mammals in Japan and *G. neoplasticum* worms in wild rats in Southeast Asia, *Gongylonema* spp. in wild mammals demonstrated diverse *cox-1* haplotypes, whereas *G. pulchrum* in domestic ruminants showed homologous *cox-1* nucleotide sequences. Although *G. nepalensis* in Sardinia and Nepal had distinct *cox-1* nucleotide sequences (Varcasia et al. 2017), those of the worms from domestic and wild mammals on Sardinia Island showed little diversity.

3. 4 Discussion

In an earlier study from our laboratory, one juvenile *Gongylonema* worm was found in the esophageal mucosa of a feral alien Reeves's muntjac collected in June 2009 on Izu-Oshima Island. The worm was subsequently specified as *G. pulchrum* of cattle-genotype (Makouloutou et al. 2013a). In the same host species at the same locality, the present study detected a high rate (34.38%) of adult worms embedded in the esophageal mucosa in typical zig-zag fashion. These adult worms grew and developed well in Reeves's muntjacs, alien cervids recently naturalized in Japan, and their morphometrics were comparable to those of worms in cattle, not those of worms in sika deer (Table 11). Furthermore, their rDNA genotype corresponded with cattle-type, not deer-type, coincident with our previous report (Makouloutou et al. 2013a). The single juvenile worm analyzed in our previous study showed cattle-I haplotype of *cox-1* (Makouloutou et al. 2013a); three of the four adult worms examined here showed the same *cox-1* haplotype. Intriguingly, the remaining worm showed cattle -II haplotype of *cox-1*. Izu-Oshima Island is a small remote island with a limited variety of endemic and alien mammals, i.e. Japanese weasels, four species of rodent (*Apodemus speciosus*, *Mus musculus*, *Rattus rattus*, and *Rattus norvegicus*), four species of bat, Reeves's muntjacs, alien Formosan rock macaques (*Macaca cyclopis*), and alien Taiwan squirrels (*Callosciurus erythraeus*), in addition to a small

number of Holsterin Friesian cattle (c. 30 at present). As reported previously (Makouloutou et al. 2013a; Chapter I), cattle (at least in Japan and China) exhibit *G. pulchrum* of two *cox-1* haplotypes (cattle-I and cattle-II) and zoo animals such as squirrel monkeys display *G. pulchrum* of *cox-1* cattle-I haplotype. The origin of *G. pulchrum* detected in feral alien Reeves's muntjacs on Izu-Oshima Island (rDNA of cattle-genotype and *cox-1* cattle-I and cattle-II haplotypes) is unclear. It is not known whether these animals brought the parasite from their original endemic region such as Taiwan or mainland China, or acquired the parasite after introduction to the zoo facility in 1938 and/or naturalization on the island in the 1980s. In the late 1980s, the population of Holstein Friesian breed reached c. 1200 on the island, and it is speculated that frequent exposures of feral Reeves's muntjacs to the *G. pulchrum* infective stage in the environment might occur. In order to resolve this issue, it is vital to ascertain the infection status of *Gongylonema* sp(p). in this small cervid species from its original endemic regions and determine the rDNA genotype and mtDNA *cox-1* haplotype of the worms, if any are indeed present.

Based on 369-bp long nucleotide sequences, we previously demonstrated a remarkable diversity of *cox-1* haplotypes of *G. pulchrum* from wild mammals such as sika deer, wild boars, and Japanese macaques (Makouloutou et al. 2013a; Chapter I). Similarly, a high genetic diversity of *cox-1* haplotypes of *G. neoplasticum* in rats in Southeast Asia has been demonstrated (Chapter II). The present study's records of a red fox and a wild boar as definitive hosts of *G. nepalensis* are new, although pigs and wild boars are known to be highly susceptible definitive hosts for closely related *G. pulchrum* (Ward 1916; Zinter and Migaki 1970; Eslami and Farsad-Hamdi 1992). Considering that a wide variety of mammals classified in different categories, including bears, a species of the order Carnivora (the suborder Caniformia), are

important definitive hosts for *G. pulchrum* (Kirkpatrick et al. 1986), the development of *G. nepalensis* in the oral mucosa of a red fox is not an aberrant finding. Presently, only three haplotypes of *cox-1*, with only one nucleotide substitution among them, have been noted in Sardinian *G. nepalensis* in various domestic ruminants, wild mouflon, and a red fox. It is possible that the highly homologous status of the 852-bp long *cox-1* nucleotide sequences can be partially ascribed to a special ecological feature of the location of the sample collections, i.e. an island. Although it is undetermined how *G. nepalensis* was distributed in mammals on Sardinia, dramatically reduced genetic variability of organisms on this small island is a well-known phenomenon (Nei et al. 1975; Frankham 1997). As reported in Varcasia et al. (2017), the nucleotide identities of 369-bp long *cox-1* sequences of *G. nepalensis* in ruminants from Nepal and Sardinia were rather low, ranging from 92.95% to 93.22%. In contrast, the rDNA nucleotide sequences, including the ITS regions, were found to be highly similar (Table 12). We therefore speculate that a fairly high genetic diversity of *cox-1* is a likely factor in the original endemic area(s) of *G. nepalensis*, but are unable to propose any candidate localities at present. Genetic surveys of *cox-1* nucleotide diversity worldwide may enable us to establish the true biogeography of *G. pulchrum* (believed to be cosmopolitan in distribution) and *G. nepalensis* (currently identified at limited localities and mainly from domestic ruminants). Such studies could indicate the locale where their ancestor originated before dispersion to different continents.

Another potential important application of molecular genetic analyses of *Gongylonema* worms is to aid the clarification of the transmission dynamics of the parasites in the natural environment. We do not know the reason why domestic cattle and wild mammals barely share the same genetic lineages of *G. pulchrum* in Japan. *G. pulchrum* of deer-genotype has not been

seen in cattle, nor has *G. pulchrum* of cattle-genotype been seen in wild mammals, with the exception of sika deer in Hokkaido which on a rare occasion were found to be infected with *G. pulchrum* of cattle-genotype (Makouloutou et al. 2013a; Chapter I). As discussed previously (Makouloutou et al. 2013a), populations of sika deer in Hokkaido, one of the four main islands of Japan, experienced dramatic reduction, nearly extinction, around 150 years ago as a result of overexploitation. Heavy snowfalls during the winters of 1879 and 1903 also took their toll on population numbers (Inukai 1952; Nabata et al. 2004). Thus, it is possible that this severe population decline of host sika deer induced the extinction of their original *G. pulchrum* of deer-genotype and may explain the scarcity of *G. pulchrum* parasitism in Hokkaido sika deer (Kitamura et al. 1997) in contrast to its high prevalence in sympatric cattle (Suzuki et al. 1992). Taking into account that Hokkaido sika deer were found to be parasitized only rarely with *G. pulchrum* and the worms were demonstrated to be cattle-genotype, not deer-genotype (Makouloutou et al. 2013a), sika deer appear to exhibit a degree of resistance to *G. pulchrum* of cattle-genotype in spite of the high likelihood of their ingestion of third-stage larvae. In contrast to this situation, feral alien Reeves's muntjacs appear to be sufficiently susceptible to *G. pulchrum* of cattle-genotype. As discussed above, the origin of *G. pulchrum* of *cox-1* cattle-I and cattle-II haplotypes in this alien mammalian species is unclear. In other words, it is uncertain whether *G. pulchrum* in feral alien Reeves's muntjacs on Izu-Oshima Island is indigenous or newly acquired after introduction to Japan. The growing accumulation of the genetic backgrounds of *Gongylonema* spp. in various animals worldwide will facilitate our understanding of typical frameworks of speciation and geographical dispersion of spirurid nematodes with a suspected wide host specificity. It will also help to define their local

transmission dynamics, which will be of consultative use when considering other host -parasite relationships and understanding parasite transmission schema.

GENERAL DISCUSSION AND CONCLUSION

The genus *Gongylonema* Molin, 1857 (Nematoda: Spirurida: Gongylonematidae) has a long history, attracting many researchers due to its unique morphological character, distinctive verruciform cuticular protrusions of anterior part of the body, and habits in the epithelial layer of final host (stratified flattened epithelium) with parasitism in a zig-zag fashion, difficulty of specific identification attributed to morphometric variation in different host animals, and zoonotic potential to human beings. Human gongylonemiasis has been recorded from many countries, with more than 150 cases worldwide since the first reported case in 1864 in Italy (Xu et al., 2000; Haruki et al., 2005; Allen and Esquela-Kerscher, 2013; Pesson et al., 2013). Different specific names were given to the causative nematodes of human gongylonemiasis, such as *G. labiale* (Pane, 1864), *G. subtile* (Alessandrini, 1914), and *G. hominis* (Stiles, 1921), all of which became currently junior synonyms of *G. pulchrum* Molin, 1857 (see Table 1). Similarly, *G. spirale* (Molin, 1857) from fallow deer, *G. contortum* (Molin, 1860) from bears, *G. scutatum* (Leuckart, 1873) from horses, cattle, goats and sheep, *G. confusum* (Sonsion, 1896) from horses, and *G. ransomi* (Chapin, 1922) from pigs became currently junior synonyms of *G. pulchrum* (Yamaguti, 1961; Skrjabin et al. 1967). To date, approximately 40 nominal species have been classified in the genus (Sato 2009; Kinsella et al. 2016; Chapter 1).

In Japan, the distribution of *G. pulchrum* was for the first time noticed in the late 1980' (Kudo et al. 1992; Suzuki et al. 1992). Kudo et al. (1992) and Suzuki (1992) recorded high prevalence of *G. pulchrum* in the esophagus of cattle in the northern part of Japan, i.e. Aomori, Iwate, Akita, and Hokkaido Prefectures with a prevalence of 18.4% and intensity of 1–318 (predominantly 1–5) worms or a prevalence of 8.3% and intensity of 1–180 worms (predominantly 1–20), respectively. After their works, *G. pulchrum* infection has been recorded

in Japanese macaques (Uni et al. 1992, 1994; Makouloutou et al. 2013a), sika deer (Yokohata et al. 1993; Kitamura et al. 1997; Makouloutou et al. 2013a), squirrel monkeys in captivity (Sato et al. 2005; Makouloutou et al. 2013a), Japanese wild boars (Makouloutou et al. 2013a), feral alien Reeves's muntjacs (Makouloutou et al. 2013a; Chapter III), and human patients (Haruki et al. 1997; Hatta et al. 2005; Hara 2010). Infection of these final hosts occur by accidental ingestion of infected dung beetles or water contaminated with third stage larvae that have emerged from dead dung beetles (Lichtenfels 1971; Kirkpatrick et al. 1986; Anderson 1992; Kudo et al. 1996). In Japan, Kudo et al. (1996) examined 16422 dung beetles collected from grassland for cattle in Aomori Prefecture, and reported a high prevalence of natural infection in dung beetles, such as six *Aphodius* spp., six *Copris* spp., *Liatongus phanaeoides*, and *Onthophagus lenzii*.

Since *G. pulchrum* has been recorded in a wide spectrum of mammals such as cattle, sheep, goats, camels, pigs, equines, cervids, rodents, bears and primates, we previously believed that susceptible mammals living sympatrically might have a similar chance to be parasitized through accidental ingestion of the third-stage larvae of this spirurid nematode. In addition, a high prevalence of the parasite in local populations of sika deer in localities where stock farming is prosperous seemed to support this view (Yokohata and Suzuki 1993). Based on molecular genetic analyses of the rDNA, particularly the ITS regions, and partial *cox-1* region of mtDNA of *G. pulchrum* worms isolated from multiple mammalian hosts, Makouloutou et al. (2013a) demonstrated, however, that cattle and wild endemic mammals (sika deer, wild boars, and Japanese macaques) have distinct transmission cycles of the species. Namely, they demonstrated two major *cox-1* haplotypes of *G. pulchrum* were found in cattle, distinct from any haplotypes in wild mammals, although the mechanism underlying this phenomenon, i.e.

separated transmission cycles in domestic and wild mammals, is unknown. One exception is Hokkaido sika deer, from which *G. pulchrum* of a cattle-I *cox-1* haplotype homologue was isolated (Makouloutou et al. 2013a). Makouloutou et al. (2013a) speculated the reason for this exceptional finding as follows: '1) Hokkaido sika deer have a history of drastic population decline/near extinction occurring more than 100 years ago as a result of the overexploitation and overharvesting of deer, beginning in 1869, and heavy snowfalls during the winters of 1879 and 1903 (Inukai, 1952; Nabata et al., 2004); 2) this severe population decline history of host sika deer induced the extinction of their original *G. pulchrum*, which was followed by recent re-acquisition of the parasite common in the Holstein–Friesian breed in dairy farms popular in Hokkaido.

Although Halajian et al. (2010) and Makouloutou et al. (2013a) reported for the first time nucleotide sequences of the rDNA and partial *cox-1* mtDNA (369-bp) of *G. pulchrum* of various origin, but collected mostly in Japan, it was unclear yet whether a spectrum of nucleotide sequences provided by these previous studies represented a majority of *G. pulchrum* individuals worldwide. Then, I planned first to collect *G. pulchrum* worms in the Chinese continent as described in Chapter I. *G. pulchrum* worms collected from cattle in Alashan League, Inner Mongolia, China, had almost identical rDNA nucleotide sequences and two *cox-1* haplotypes (cattle-types I and II) as seen in *G. pulchrum* from the cattle in Japan, supporting an idea that *G. pulchrum* worms in domestic animals like cattle with cosmopolitan distribution might be a homologous population with an almost identical rDNA nucleotide sequences including the ITS regions. These worms showed again two *cox-1* haplotypes, and a proper elucidation of the origin of such different *cox-1* haplotypes of *G. pulchrum* worms in cattle remains to be challenged in the future.

Makouloutou et al. (2013b) found a lineage of *Gonglyonema* worms from water buffaloes in Nepal, which showed the closest morphological identity with *G. pulchrum* except for a proportion of the left spicule length to the entire worm length. Genetically the worms showed apparent uniqueness of the rDNA and *cox-1* nucleotide sequences from those of *G. pulchrum* isolated from various mammals in Japan (Makouloutou et al. (2013b). Up to that time, interspecific genetic differences among spiruruid nematodes had not been shown, and the significance of nucleotide sequence differences between *G. pulchrum*-like worms in water buffaloes in Nepal and *G. pulchrum* were unclear. To clarify interspecific nucleotide sequences, the rDNA and *cox-1* nucleotide sequences of rondent *Gongylonema* worms, *G. aegypti* from spiny mice in Egypt and *G. neoplasticum* from a black rat in Okinawa Island, Japan, were analyze. The genetic diversities of three distinct *Gongylonema* spp. and their possible intraspecific genetic variation may allow us to resolve the taxonomic position of *Gongylonema* spp. which display few obvious morphological differences from their congeners. Consequently, the *Gongylonema* isolate from water buffaloes in Nepal reported by Makouloutou et al. (2013b) is concluded to be a new species, and new species *G. nepalensis* is erected for it (Chapter I). Recently, this species has been recorded from a variety of mammals on Sardinia Island, Italy (Varcasia et al. 2017).

Makouloutou's study based on the rDNA and *cox-1* of mt DNA of *G. pulchrum* from cattle, sika deer, wild boars, Japanese macaques, a feral Reeves' muntjac and captive squirrel monkeys in Japan indicated the life cycle of *G. pulchrum* of domestic and wild animals is separated at least in Japan (Makouloutou et al. 2013a). To explore possible biogeographical separation of another rodent *Gongylonema* worms, i.e. *G. neoplasticum* in murids, I conducted a cooperative research with Dr. Ribas, University of Barcelona, and his cooperative researchers in the

Southeast Asia such as Thailand, Cambodia, Philippines, and Indonesia as written in Chapter II. This is the first study of morphological and genetic characterization of *G. neoplasticum* from 127 rats in Southeast Asia where is the original habitat of *Rattus* spp., predominant hosts for this species, showing 24 *cox-1* haplotypes regardless of parasite origin. It is likely to be nearly a maximum spectrum of genetic diversity of this species, because the parasite collection was conducted in its original endemic area. The previous data of *cox-1* haplotype of *G. neoplasticum* from a black rat in Okinawa, mentioned in Chapter I, was identical to the major *cox-1* haplotype in this study (Chapter II). Similarly, *G. neoplasticum* isolates could be at the similar situation to this case, since this spirurid species has distributed around the world with global dispersion of its rodent hosts beyond Asia as a consequence of recent global trade (Chapter II). As known, *Gongylonema* spp. sometimes show morphological variation, especially when the number of specimens is a few, molecular analysis is great important and reliable and may help to detect substantial specific diversities (Kinsella et al. 2016).

In Chapter III, I focused on the genetic diversity of *G. pulchrum* from wild animals in Japan and *G. nepalensis* from wild animals in Sardinia in Italy based on longer *cox-1* sequences (852-bp). Intriguingly, *cox-1* haplotypes of *G. pulchrum* from feral alien Reeves's muntjacs on Izu-Oshima Island, Tokyo, were identical to cattle-haplotypes; namely, that of three of four worms from this animal was identical to cattle-I haplotype and the other was identical to cattle-II haplotype. Newly obtained *G. nepalensis* worms from goat and red fox were highly homologous with previous *G. nepalensis* from domestic ruminants and wild mouflon in Sardinia in Italy (Varcasia et al. 2017). As mentioned in Chapter III, *G. nepalensis* is absolutely popular in Sardinia, and the lowest genetic diversity of worms might be explained by the

phenomenon that the genetic variation dramatically reduced in the small island (Nei et al. 1975; Frankham 1997).

My studies mentioned through Chapters I to III have clarified outlines of possible genetic diversities of *G. pulchrum*, *G. nepalensis*, and *G. neoplasticum* using a variety of sources. Along with genetic data on *G. aegypti*, I believe that my studies on *Gongylonema* worms can provide a research platform for genetic differentiation of *Gongylonema* spp., followed by further characterization of different species of the genus. In addition, I have shown the utility of genetic characters of the worm in discussing transmission dynamics of the worms in nature. Since our understanding of *Gongylonema* worms is still partial from viewpoints of specific diversity and transmission dynamics in nature, further investigative challenging on the worms are necessary yet.

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TABLES

Table 1 List of representative *Gongylonema* spp. which became junior synonyms of *G. pulchrum* at present[a].

Species name	Host	Locality
G. spirale (Molin, 1857)	Fallow deer	Europe
G. contortum (Molin, 1860)	Bears	Europe
G. labiale (Pane, 1864)	Human patient	Italy
G. scutatum (Leuckart, 1873)	Horses, cattle, goats and sheep	Europe
G. confusum (Sonsino, 1896)	Horses	Egypt
G. subtile (Alessandrini, 1914)	Human patient	Italy
G. hominis (Stiles, 1921)	Human patient	North America
G. ransomi (Chapin, 1922)	Pigs	Europe

[a] After Yamaguti (1961) and Skrjabin et al. (1967).

Table 2 Recovery of *G. pulchrum* from the esophagus of cattle and goats in Alashan League, Inner Mongolia.

Host	No. of animals examined	No. of positive animals (%)	Intensity	Deposited specimens[a]
Cattle	68	49 (72.1%)	1—13 (average, 5.3)	NSMT-As3974–4022
Goat	19	9 (47.4%)	1—7 (average, 4.1)	NSMT-As3969–3973

[a] Specimen ID numbers at the National Museum of Nature and Science, Tokyo.

Table 3 Comparison of measurements of *Gongylonema s*pecimens collected from ruminants (in mm)[a]

Species	*G. pulchrum*	*G. pulchrum*	*G. pulchrum*	*G.* cf. *pulchrum*[b]
Host	Cattle	Cattle	Cattle	Buffalo
Locality	China	Japan	Iran	Nepal
Reference	The present study	Sato (2009)	Halajian et al. (2010)	Makouloutou et al. (2013b)
Male worm				
Number of worms examined	6	6	6	8
Body length	30.9-52.7 (40.4±7.1)	30.7-44.9 (36.8±5.8)	36.7-48.6 (41.9±4.8)	37.0-46.6 (41.1±3.5)
Max. body width	0.12-0.23 (0.21±0.04)	0.26-0.30 (0.28±0.02)	0.22-0.26 (0.24±0.02)	0.20-0.28 (0.23±0.03)
Pharynx	0.045-0.056 (0.052±0.005)	0.048-0.056 (0.052±0.004)	0.045-0.056 (0.048±0.005)	0.039-0.050 (0.044±0.004)
Oesophagus	5.35-6.96 (6.08±0.69)	4.50-6.64 (5.58±0.43)	4.96-6.08 (5.58±0.43)	4.73-7.80 (6.22±1.08)
Muscular portion	0.59-0.78 (0.68±0.08)	0.29-0.51 (0.43±0.08)	0.55-0.65 (0.61±0.04)	0.45-0.63 (0.56±0.06)
Glandular portion	4.77-6.17 (5.40±0.63)	4.02-6.21 (5.05±0.89)	4.40-5.47 (4.97±0.43)	4.28-6.62 (5.43±0.87)
Cervical lateral papillae[c]	0.121-0.169 (0.145±0.026)	0.112-0.176 (0.143±0.024)	0.134-0.171 (0.150±0.013)	0.122-0.169 (0.144±0.019)
Nerve ring[c]	0.175-0.366 (0.293±0.065)	0.220-0.288 (0.259±0.031)	0.266-0.322 (0.298±0.020)	0.256-0.372 (0.296±0.039)
Excretory pore[c]	0.462-0.637 (0.514±0.071)	0.352-0.496 (0.432±0.065)	0.495-0.599 (0.537±0.039)	0.461-0.583 (0.512±0.046)
Left spicule	14.68-21.75 (17.77±2.61)	14.19-20.36 (17.60±2.20)	10.60-27.86 (18.87±6.46)	5.89-7.94 (7.02±0.81)
Relative length of left spicule / body length	40.3-49.6 (44.2) %	39.5-64.1 (48.9) %	21.8-65.6 (46.3) %	15.6-21.1 (18.4) %
Right spicule	0.135-0.186 (0.159±0.020)	0.096-0.160 (0.132±0.024)	0.137-0.168 (0.157±0.014)	0.111-0.153 (0.133±0.014)
Gubernaculum	0.079-0.130 (0.104±0.019)	0.072-0.104 (0.083±0.011)	0.109-0.140 (0.130±0.018)	0.072-0.125 (0.099±0.015)
Number of precloacal papillae	6	4 or 5	5 or 6	5 or 6
Number of postcloacal papillae	5	5 or 6	5 or 6	5
Tail length	0.282-0.361 (0.323±0.036)	0.240-0.300 (0.270±0.028)	0.172-0.336 (0.275±0.062)	0.267-0.417 (0.325±0.048)
Female worm				
Number of worms examined	6	6	6	8
Body length	70.8-89.8 (79.6±7.7)	67.9-85.1 (78.5±7.2)	68.5-107.3 (82.4±14.4)	60.0-91.6 (72.7±10.0)
Max. body width	0.28-0.35 (0.30±0.03)	0.30-0.34 (0.33±0.02)	0.29-0.38 (0.32±0.04)	0.24-0.41 (0.33±0.05)
Pharynx	0.048-0.056 (0.052±0.004)	0.040-0.072 (0.057±0.012)	0.045-0.064 (0.055±0.007)	0.050-0.069 (0.060±0.008)
Oesophagus	6.28-7.66 (7.14±0.58)	6.75-7.41 (7.12±0.24)	6.46-8.39 (7.58±0.72)	7.58-9.89 (8.91±0.84)
Muscular portion	0.65-0.83 (0.70±0.07)	0.50-0.86 (0.68±0.14)	0.63-0.88 (0.80±0.10)	0.47-0.81 (0.62±0.10)
Glandular portion	5.62-7.00 (6.44±0.56)	6.16-6.64 (6.43±0.19)	5.83-7.52 (6.77±0.63)	6.93-9.58 (8.29±0.82)
Cervical lateral papillae[c]	0.152-0.214 (0.177±0.023)	0.124-0.160 (0.145±0.015)	0.168-0.224 (0.189±0.020)	0.150-0.192 (0.167±0.017)
Nerve ring[c]	0.338-0.417 (0.369±0.027)	0.312-0.400 (0.352±0.034)	0.324-0.453 (0.382±0.049)	0.272-0.417 (0.343±0.055)
Excretory pore[b]	0.468-0.676 (0.586±0.092)	0.480-0.640 (0.568±0.067)	0.632-0.862 (0.743±0.087)	0.539-0.722 (0.608±0.069)
Vulva[d]	3.55-5.24 (4.17±0.62)	2.72-4.08 (3.15±0.50)	2.69-5.18 (3.63±0.96)	3.42-4.58 (3.91±0.40)
Tail length	0.259-0.377 (0.303±0.044)	0.260-0.340 (0.307±0.033)	0.246-0.414 (0.307±0.058)	0.167-0.272 (0.216±0.031)
Egg	0.054-0.060 (0.056±0.002) x 0.032-0.036 (0.033±0.001)	0.056-0.060 (0.059±0.002) x 0.032-0.036 (0.035±0.002)	0.058-0.060 (0.059±0.001) x 0.032-0.034 (0.033±0.001)	0.055-0.058 (0.057±0.001) x 0.030-0.032 (0.031±0.001)

[a] Values clearly deviated from those of other groups are encased by lines.

[b] In the present study, we propose a new species name, *Gongylonema nepalensis* n. sp., for this *Gongylonema* lineage.

[c] From the anterior end.

[d] From the posterior end.

Table 4 Nucleotide changes observed in the rDNA of *Gongylonema pulchrum* of different origins.

Host	Locality	DDBJ/EMBL/GenBank accession no.	No. of worms examined	Nucleotide position where any changes in rDNA are observed[a]														Genotype
				18S	ITS1							ITS2[b]				28S		
				13	51 - 59	71	122 - 132	302 -			389	66-67	68-			542	897	
Deer																		
	Hyogo Prefecture, Japan	AB495394 - AB495396	1	A	(CAA) x 3	C	(GA) x 5	(A) x 12 - 19	(CA) x 5	(TA) x 1	(CA) x 4	A	TT	(GCT) x 8	—	—	C	T	Deer-type
	Hyogo Prefecture, Japan	AB495397 - AB495400	1	A	(CAA) x 3	C	(GA) x 5	(A) x 15 - 18	(CA) x 4 - 5	(TA) x 1-2	(CA) x 4	A	TT	(GCT) x 8	—	—	C	T	Deer-type
	Hyogo Prefecture, Japan	AB645978 - AB645982	4		(CAA) x 3	C	(GA) x 5	(A) x 13 - 20	(CA) x 2 - 6	(TA) x 1	(CA) x 4	A	TT	(GCT) x 6 - 17	—	—	C	T	Deer-type
	Kagoshima Prefecture, Japan	AB646055 - AB646058	1	G	(CAA) x 3	C	(GA) x 5	(A) x 14 - 20	(CA) x 4 - 7	(TA) x 1	(CA) x 4	A	TT	(GCT) x 5-8	—	—	A	C	Deer-type
	Hokkaido Prefecture, Japan	AB646059 - AB646063	2	G	(CAA) x 3	C	(GA) x 5	(A) x 12 - 13	(CA) x 3	(TA) x 0-1	(CA) x 5-8	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	C	Cattle-type
Wild boar																		
	Hyogo Prefecture, Japan	AB646064 - AB646094	7	G	(CAA) x 3	C	(GA) x 5	(A) x 10 - 20	(CA) x 2 - 6	(TA) x 1	(CA) x 4	A	TT	(GCT) x 6-16	—	—	C	C	Deer-type
Japanese macaque																		
	Nara Prefecture, Japan	AB646095 - AB646105	2		(CAA) x 3	C	(GA) x 5	(A) x 15-19	(CA) x 4-5	(TA) x 1	(CA) x 5	A	TT	(GCT) x 8	—	—			Deer-type
Squirrel monkey																		
	Captive in Japan	AB495401 - AB495402	2	G	(CAA) x 3	C	(GA) x 4	(A) x 12-13	(CA) x 3	(TA) x 1	(CA) x 7-8	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
Cattle																		
	Okayama or Tottori Prefecture, Japan	AB513707 - AB513710	1	G	(CAA) x 3	C	(GA) x 4	(A) x 14-15	(CA) x 3	(TA) x 1	(CA) x 7-9	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Oita Prefecture, Japan	AB513711 - AB513718	1	G	(CAA) x 3	C	(GA) x 4	(A) x 13-17	(CA) x 3-4	(TA) x 1	(CA) x 4-10	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Kagoshima Prefecture, Japan	AB513719 - AB513723	1	A	(CAA) x 3	C	(GA) x 4	(A) x 12-14	(CA) x 3-4	(TA) x 1	(CA) x 6-8	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Iran	AB495389 - AB495393	1	A	(CAA) x 3	C	(GA) x 4	(A) x 12-14	(CA) x 3	(TA) x 1	(CA) x 3-8	A	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Inner Mongolia, China	**LC026017-LC026028**	**12**	**G**	**(CAA) x 2-4**	**C/T**	**(GA) x 4-7**	**(A) x 11-18**	**(CA) x 3-4**	**(TA) x 1**	**(CA) x 3-9**	**A/T**	**CT**	**(GCT) x 2**	**GCG**	**(GCT) x 2**	**A**	**T**	**Cattle-type**

[a] Nucleotide position is expressed relative to the rDNA sequence of *G. pulchrum* collected from a deer caught on Awaji Island (DDBJ/EMBL/GenBank accession no. AB495394). Gaps are indicated by '-' and blanks indicate no data.

[b] Nucleotide arrangements in boxes are typical for cattle-type ITS2 sequences, distinct from those of deer-type ITS2 ones.

Table 5 Nucleotide substitutions observed in the *cox-1* mtDNA of *Gongylonema pulchrum* of different origins.

Host	Locality	DDBJ/EMBL/GenBank accession no.	Nucleotide position where any changes in COI mtDNA are observed[a]																
			33	46	69	76	84	114	117	132	165	207	216	234	327	337	351	354	
Deer																			
	Hyogo Prefecture, Japan	AB513724, AB646110, AB646111	A	T	T	G	G	A	T	T	T	C	G	G	T	C	G	A	
	Hyogo Prefecture, Japan	AB646112	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	
	Hyogo Prefecture, Japan	AB646113 - AB646114	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	
	Hyogo Prefecture, Japan	AB646115	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	
	Wakayama Prefecture, Japan	AB646116 - AB646118	G	•	C	•	•	G	•	•	•	•	•	•	•	T	A	•	
	Kochi Prefecture, Japan	AB513725	•	•	•	A	•	•	•	•	•	•	•	•	•	T	A	•	
	Kochi Prefecture, Japan	AB646119	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	
	Hokkaido Prefecture, Japan	AB646120	•	C	•	•	•	•	C	C	•	T	A	A	•	T	•	•	
Wild boar																			
	Hyogo Prefecture, Japan	AB646121 - AB646122	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	
	Hyogo Prefecture, Japan	AB646123	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
	Hyogo Prefecture, Japan	AB646124	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	
	Hyogo Prefecture, Japan	AB646125	•	•	C	•	•	•	•	•	•	•	•	•	•	T	•	•	
Japanese macaque																			
	Nara Prefecture, Japan	AB646126 - AB646129	G	•	C	•	•	G	•	•	•	•	•	•	•	T	A	•	
Cattle																			
	Japan	AB513727	•	C	•	•	•	•	•	C	•	T	A	A	•	T	•	•	
	Inner Mongolia, China	LC026034, LC026041, LC026042	•	C	•	•	•	•	C	C	•	T	A	A	•	T	•	•	'cattle-I' haplotype
	Iran	AB513726	•	C	•	•	•	•	C	C	•	T	A	A	•	T	•	•	
	Japan	AB513728	•	C	•	•	•	•	C	C	C	T	•	A	C	T	•	G	'cattle-II' haplotype
	Inner Mongolia, China	LC026035 - 026040, LC026043 - 026045	•	C	•	•	•	•	•	C	C	T	•	A	C	T	•	G	
Squirrel monkey																			
	Saimiri boliviensis captive in Japan	AB513729	•	C	•	•	•	•	C	C	•	T	A	A	•	T	•	•	
	Saimiri sciureus captive in Japan	AB513730	•	C	•	•	•	•	C	C	•	T	A	A	•	T	•	•	

[a]Nucleotide position is expressed relative to the 5'-end of *cox-1* sequence of *G. pulchrum* collected from a deer caught on Awaji Island, Hyogo Prefecture, Japan (DDBJ/EMBL/GenBank accession no. AB513724). Dots denote an identical base to that of *G. pulchrum* collected from the deer in Hyogo. Nucleotide arrangements encased by lines resemble clearly each other.

Table 6 *Gongylonema neoplasticum* worms examined in the present study[a]

Host rodent speceis	Thailand	Cambodia	Laos	Philippines	Indonesia	Total
Rattus norvegicus	29 (25)	2 (1)			13 (4)	44 (30)
Rattus exulans	10 (1)	25 (7)	2 (1)			37 (9)
Rattus tanezumi	10 (4)	20 (3)		1 (1)		31 (8)
Rattus andamanensis	2 (2)					2 (2)
Rattus sp.			1 (1)	1 (1)		2 (2)
Maxomys surifer	5 (3)	2 (0)				7 (3)
Niviventer fulvescens		3 (0)				3 (0)
Berylmys bowersi	1 (1)					1 (1)
Total	57 (36)	52 (11)	3 (2)	2 (2)	13 (4)	127 (55)

[a] Number of studied worms from different rodent individuals except for Indonesia (Number of worms reactive to PCR amplification of the rDNA and/or *cox-1* mtDNA fragments).
In Indonesia, 17 worms were collected from four rats; nine and four worms from two rats were were used for morphological and molecular genetic analyses.

Table 7 Comparison of measurements of *Gongylonema neoplasticum* specimens collected from murids and the European rabbit (in mm)

Host	*Rattus* spp. (*R. norvegicus, R. exulans, R. tanezumi*), *Maxomys surifer*	*R. norvegicus*	*Rattus* spp. (*R. norvegicus, R. rattus*), *Mus musculus*	*Rattus norvegicus*? (Experimental white rat)	*Oryctolagus cuniculus*
Locality	Thailand, Cambodia	Indonesia (Surabaya)	Denmark	Taiwan	Portugal
Reference	Present study	Present study	Fibiger & Ditlevsen (1914)	Yokogawa (1925)	Eira et al. (2006)
Male worm	(n=6)	(n=6)	(n=?)	(n=10)	(n=10)
Body length	7.6–12.5 (9.6)	13.5–15.4 (14.6)	15–20	9–16 (12.1)	11.6–16.0 (13.3)
Max. body width	0.12–0.17 (0.15)	0.16–0.20 (0.19)	0.110–0.130	0.095–0.15 (0.13)	0.14–0.22 (0.18)
Length of pharynx	0.029–0.061 (0.044)	0.053–0.063 (0.058)	—	0.040–0.070 (0.049)	0.028–0.039 (0.034)
Length of esophagus	1.38–2.40 (1.91)	2.99–3.50 (3.27)	2.8	2.17–3.09 (2.72)	—
Muscular esophagus	0.20–0.39 (0.29)	0.40–0.47 (0.42)	—	0.225–0.434 (0.335)	0.41–0.49 (0.45)
Grandular esophagus	1.18–2.04 (1.62)	2.58–3.08 (2.84)	—	1.8–2.8 (2.36)	2.67–2.99 (2.88)
Nerve ring[a]	0.12–0.20 (0.17)	0.19–0.25 (0.22)	—	—	0.21–0.26 (0.23)
Left spicule	0.55–0.85 (0.72)	0.70–0.77 (0.74)	0.528	0.61–0.63 (0.62)	0.45–0.66 (0.56)
Relative length to the body (%)	5.3–11.2 (7.7)	4.5–5.5 (5.1)	—	—	—
Right spicule	0.087–0.103 (0.096)	0.075–0.106 (0.092)	0.093	0.080–0.094 (0.086)	0.108–0.126 (0.117)
Gubernaculum	0.048–0.059 (0.054)	0.034–0.064 (0.054)	—	present	0.046–0.082 (0.062)
Female worm	(n=6)	(n=3)	(n=?)	(n=10)	(n=10)
Body length	32.8–64.6 (44.8)	56.0–75.0 (65.7)	60–80	45–115 (74)	34.27–58.6 (43.36)
Max. body width	0.18–0.29 (0.23)	0.26–0.26 (0.26)	0.170–0.326	0.26–0.37 (0.32)	0.30–0.36 (0.32)
Length of pharynx	0.051–0.071 (0.059)	0.053–0.072 (0.063)	0.053	0.06–0.12 (0.074)	0.028–0.049 (0.039)
Length of esophagus	3.34–6.14 (4.88)	6.40–7.38 (6.79)	7	—	—
Relative length to the body	8.47–18.61 (11.49)	8.85–11.42 (10.42)	—	—	—
Muscular esophagus	0.45–0.69 (0.57)	0.67–0.81 (0.76)	—	0.68–1.12 (0.83)	0.51–0.73 (0.63)
Grandular esophagus	2.87–5.45 (4.31)	5.73–6.58 (6.03)	—	4.85–7.50 (?)	4.17–5.71 (4.91)
Nerve ring[a]	0.22–0.31 (0.26)	0.27–0.33 (0.30)	—	—	0.26–0.34 (0.30)
Vulva[b]	3.5–6.4 (4.8)	5.5–8.6 (7.1)	4–7	5–10 (4.80)	5.2–9.6 (5.6)
Relative length to the body (%)	9.5–11.8 (10.8)	9.8–11.4 (10.8)	10–12.5	10–12.5	13.3–19.5 (16.9)
Tail length	0.205–0.253 (0.231)	0.216–0.356 (0.288)	0.210	—	0.198–0.330 (0.276)
Egg					
Length	0.050–0.053 (0.052)	0.047–0.050 (0.048)	0.060	0.054–0.058 (0.057)	0.046–0.059 (0.053)
Width	0.032–0.035 (0.034)	0.031–0.033 (0.032)	0.040	0.034–0.037 (0.033)	0.028–0.036 (0.034)

[a] From the anterior end.

[b] From the posterior end.

Table 8 *Gongylonema neoplasticum* worms examined for the rDNA nucleotide sequences[a]

Host rodent speceis	Thailand	Cambodia	Philippines
Rattus norvegicus	#51, #76, #79, **#82**, #85, #87		
Rattus exulans		#17, **#21**, #90	
Rattus tanezumi	#36, #40, **#49**		
Rattus andamanensis	#45		
Rattus sp.			#59
Maxomys surifer	#52		
Berylmys bowersi	**#38**		

[a] Worms reactive to PCR amplification of rDNA nucleotide fragments are shown by worm ID number. Specimens expressed in bold numbers showed a few nucleotide substitutions in the 28S rDNA (see Table 9).

Table 9 Nucleotide variations in the 28S rDNA of *Gongylonema neoplasticum* of different origins[a]

Worm ID no.	DDBJ/EMBL/GenBank accession no.	Host	Locality	28S rDNA[b]			
				437	579	814	1019
#17, #51, #59	LC330994–LC330996	*R. norvegicus* , *R. exulans* , *Rattus* sp.	Thailand, Cambodia, Philippines	A	C	C	C
#21	LC330997	*Rattus exulans*	Cambodia	·	·	T	·
#38	LC330998	*Berylmys bowersi*	Thailand	·	·	T	·
#49	LC330999	*Rattus tanezumi*	Thailand	·	T	·	·
#82	LC331000	*Rattus norvegicus*	Thailand	·	·	·	T
G. neoplasticum JPN	LC026032, LC026033	*Rattus rattus*	Japan	C	·	·	·

[a] Worms with long rDNA nucleotide sequences successfully amplified by PCR are shown.

[b] Relative nucleotide position to the 28S rDNA of a Japanese isolate of *G. neoplasticum* (DDBJ/EMBL/GenBank accession no. LC026032).

Table 10 *Gongylonema neoplasticum* worms examined for the *cox-1* mtDNA nucleotide sequences

Host rodent speceis	Thailand	Cambodia	Laos	Philippines	Indonesia
Rattus norvegicus	#51, #62, #63, #65, #66, #67, #69, #70, #71, #72, #73, #75, #76, #77, #78, #79, #80, #81, #82, #83, #84, #85, #86, #87, #89	#8			#161, #162, #163, #164
Rattus exulans	#95	#2, #7, #17, #21, #96, #97, #98	#47		
Rattus tanezumi	#36, #40, #49, #53	#15, #28, #30		#57	
Rattus andamanensis	#45, #50				
Rattus sp.			#123	#59	
Maxomys surifer	#41, #42, #52				
Berylmys bowersi	#38				

Table 11 Comparison of measurements of *Gongylonema* specimens collected from ruminants (in mm)[a]

Species	*G. pulchrum*			*G. nepalensis*				
Host	Feral Reeves's muntjac	Cattle	Deer	Fox	Wild boar	Sheep	Mouflon	Buffalo
Locality	Izu-oshima Is., Japan	Japan	Japan	Sardinia Is., Italy	Sardinia Is., Italy	Sardinia Is., Italy	Sardinia Is., Italy	Nepal
Reference	Present study	Sato (2009)	Sato (2009)	Present study	Present study	Varcasia et al. (2017)	Varcasia et al. (2017)	Makouloutou et al. (2013b)
Male worm								
Number of worms examined	3	6	6	0	1	3	3	8
Body length	27.1–45.5 (36.1)	30.7–44.9 (36.8)	21.2-26.9 (24.0)	—	31.1	41.5-44.1 (42.8)	44.8-46.5 (45.6)	37.0-46.6 (41.1)
Max. body width	0.16–0.23 (0.20)	0.26–0.30 (0.28)	0.15-0.19 (0.17)	—	0.21	0.18-0.25 (0.21)	0.27-0.30 (0.29)	0.20-0.28 (0.23)
Length of oesophagus	4.67–6.05 (5.22)	4.50–6.64 (5.58)	5.01-6.37 (5.57)	—	5.24	5.36-5.84 (5.58)	6.10-6.21 (6.13)	4.73-7.80 (6.22)
Muscular oesophagus	0.44–0.54 (0.48)	0.29–0.51 (0.43)	0.49-0.54 (0.52)	—	—	—	—	0.45-0.63 (0.56)
Grandular oesophagus	4.19–5.47 (4.71)	4.02–6.21 (5.05)	4.47-5.85 (5.05)	—	—	—	—	4.28-6.62 (5,43)
Nerve ring[b]	0.247–0.266 (0.255)	0.220–0.228 (0.259)	0.282-0.382 (0.318)	—	—	—	—	0.256-0.372 (0.296)
Excretory pore[b]	0.404–0.423 (0.413)	0.352–0.496 (0.432)	0.409-0.492 (0.458)	—	0.467	0.446-0.482 (0.464)	0.475-0.537 (0.499)	0.461-0.583 (0.512)
Left spicule	10.48–23.75 (17.52)	14.19–20.36 (17.60)	6.28-7.72 (6.78)	—	6.6	8.32-8.41 (8.37)	7.75-8.87 (8.31)	5.89-7.94 (7.02)
Relative length of left spicule / body length	38.7–52.2 (47.4) %	39.5–64.1 (48.9) %	24.6-32.3 (28.4) %	—	21.2 %	19.0-20.3 (19.6) %	17.3-19.1 (18.2) %	15.6-21.1 (18.4) %
Right spicule	0.131[d]	0.096–0.160 (0.132)	0.097-0.163 (0.115)	—	0.118	0.118-0.129 (0.123)	0.122-0.130 (0.125)	0.111-0.153 (0.133)
Tail length	0.256–0.320 (0.293)	0.240–0.300 (0.270)	0.282-0.321 (0.300)	—	0.286	0.286-0.366 (0.328)	0.364-0.386 (0.374)	0.267-0.417 (0.325)
Female worm								
Number of worms examined	4	6	6	2	1	4	3	8
Body length	51.7–93.2 (69.5)	67.9–85.1 (78.5)	46.0-51.6 (43.6)	46.8 & 35.1	49.4	98.6-120.7 (106.9)	102.7-111.7 (106.5)	60.0-91.6 (72.7)
Max. body width	0.22–0.33 (0.28)	0.30–0.34 (0.33)	0.22-0.28 (0.24)	0.25 & 0.26	0.22	0.36-0.44 (0.39)	0.34-0.40 (0.37)	0.24-0.41 (0.33)
Length of oesophagus	5.66–6.80 (6.26)	6.75–7.41 (7.12)	6.86-7.72 (7.28)	6.45 & 5.57	5.76	7.25-8.12 (7.60)	8.11-8.54 (8.30)	7.58-9.89 (8.91)
Muscular oesophagus	0.64–0.68 (0.66)	0.50–0.86 (0.68)	0.55-0.77 (0.67)	—	—	—	—	0.47-0.81 (0.62)
Grandular oesophagus	4.93–6.08 (5.55)	6.16–6.64 (6.43)	6.30-6.96 (6.60)	—	—	—	—	6.93-9.58 (8.29)
Nerve ring[b]	0.308–0.468 (0.351)	0.312–0.400 (0.352)	0.327–0.421 (0.369)	—	—	—	—	0.272-0.417 (0.343)
Excretory pore[b]	0.545–0.615 (0.575)	0.480–0.640 (0.568)	0.598-0.837 (0.734)	0.498 & 0.377	0.590	0.712-0.797 (0.756)	0.760-0.828 (0.794)	0.539-0.722 (0.608)
Vulva[c]	2.39–4.70 (3.52)	2.72–4.08 (3.15)	1.88-2.99 (2.38)	3.20 & 1.57	3.01	6.23-7.98 (7.12)	7.53-14.47 (9.91)	3.42-4.58 (3.91)
Tail length	0.229–0.336 (0.282)	0.260–0.340 (0.307)	0.266-0.316 (0.281)	0.194 & 0.206	0.241	0.264-0.342 (0.310)	0.358-0.367 (0.361)	0.167-0.272 (0.216)

[a] Values clearly deviated from those of other groups are shown by a grey box.

[b] From the anterior end.

[c] From the posterior end.

[d] Measurement of one worm.

Table 12 Inter- and intra-individual nucleotide changes observed in the ITS regions of rDNA of *Gongylonema nepalensis* of different origins

Location	Host origin	DDBJ/EMBL/ GenBank accession no.	ITS 1[a]													ITS 2[a]	
			46—			65	75	76—84	145	174	261	317—			329—	210	221/222
Nepal																	
	water buffalo	AB646107	(A) x12	(G) x0	(A) x0	A	G	CTGCTGTCA	A	G	C	(A) x12	(G) x0	(A) x0	(CA) x6	T	−−−
Sardinia																	
	cattle, sheep, goat, mouflon	LC278392	(A) x10	(G) x1	(A) x3	C	A	−−−−−−−−−	T	G	A	(A) x7	(G) x1	(A) x4	(CA) x6	T	GTG
	cattle, fox	LC3887450, LC388751	(A) x9–10	(G) x1	(A) x3	C	A	−−−−−−−−−	T	G	A	(A) x7–12	(G) x0–1	(A) x0–4	(CA) x6	T	GTG
	cattle, sheep, goat, mouflon, fox	LC388742	(A) x12–15	(G) x0	(A) x0	C	A	−−−−−−−−−	T	G	A	(A) x7	(G) x1	(A) x4	(CA) x6	T	GTG
	cattle	(Unpublished)	(A) x14	(G) x0	(A) x0	C	A	−−−−−−−−−	T	G	A	(A) x12	(G) x0	(A) x0	(CA) x6	T	GTG
	cattle, sheep, goat	(Unpublished)	(A) x10	(G) x1	(A) x3	C	A	−−−−−−−−−	T	G	A	(A) x7	(G) x1	(A) x4	(CA) x6	T	−−−
	goat	LC388744–LC388746	(A) x10–15	(G) x0–1	(A) x0–3	C	A	−−−−−−−−−	T	G	A	(A) x7	(G) x1	(A) x2–4	(CA) x6–7	G	−−−
	mouflon, fox	LC388747–LC388749	(A) x12–16	(G) x0	(A) x0	C	A	CTGCTGCTA	T	G/A	A	(A) x11–12	(G) x0	(A) x0	(CA) x6–8	T	GTG
	goat	LC388743	(A) x14	(G) x0	(A) x0	C	A	CTGCTGCTA	T	G	A	(A) x7	(G) x1	(A) x4	(CA) x6	G	−−−

[a] Nucleotide position is expressed relative to each region of the rDNA sequence of *G. nepalensis* collected from water buffaloes in Nepal (DDBJ/EMBL/GenBank accession no. AB646107). Nucleotide unit for repeats is shown in parentheses. '—' denotes no nucleotide.

FIGURES

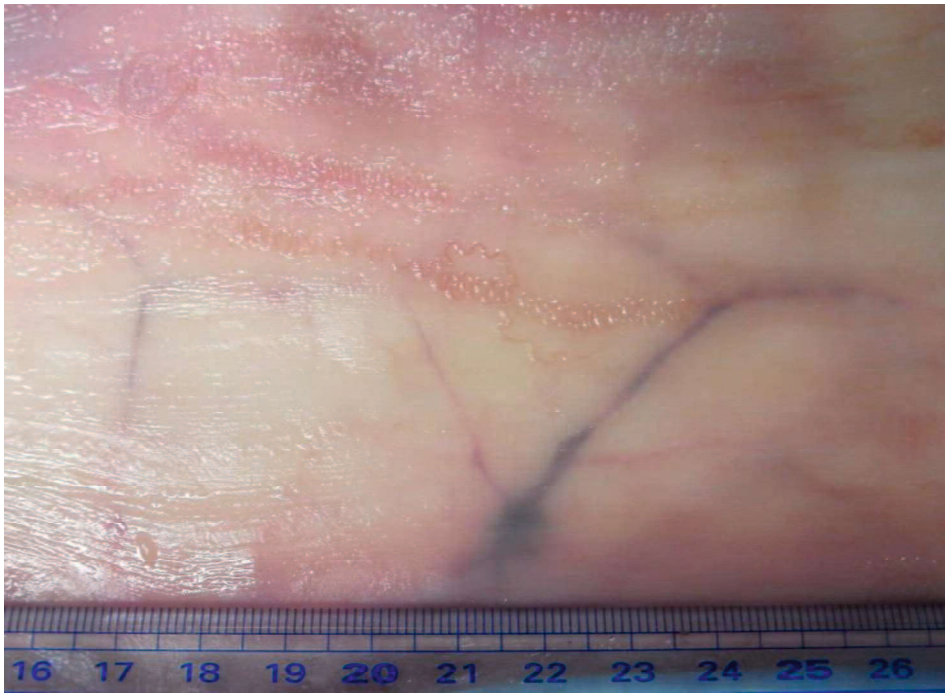

Figure 1 Adult *Gongylonema pulchrum* worms embedded in the epithelium of the esophageal mucosa of cattle in a zig-zag pattern.

Figure 2 Light microscopic view of the anterior part (esophageal part) of adult female *Gongylonema pulchrum* worm.

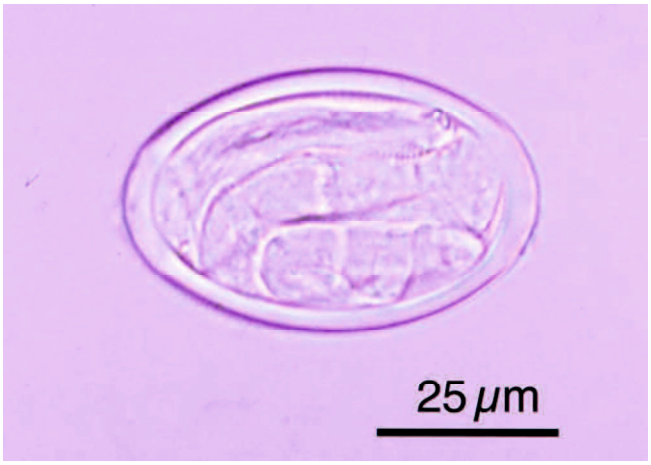

Figure 3 Intrauterine *Gongylonema pulchrum* egg, with the first-stage larvae, which has cephalic hooklets and circular rows of minute spines in its anterior portion.

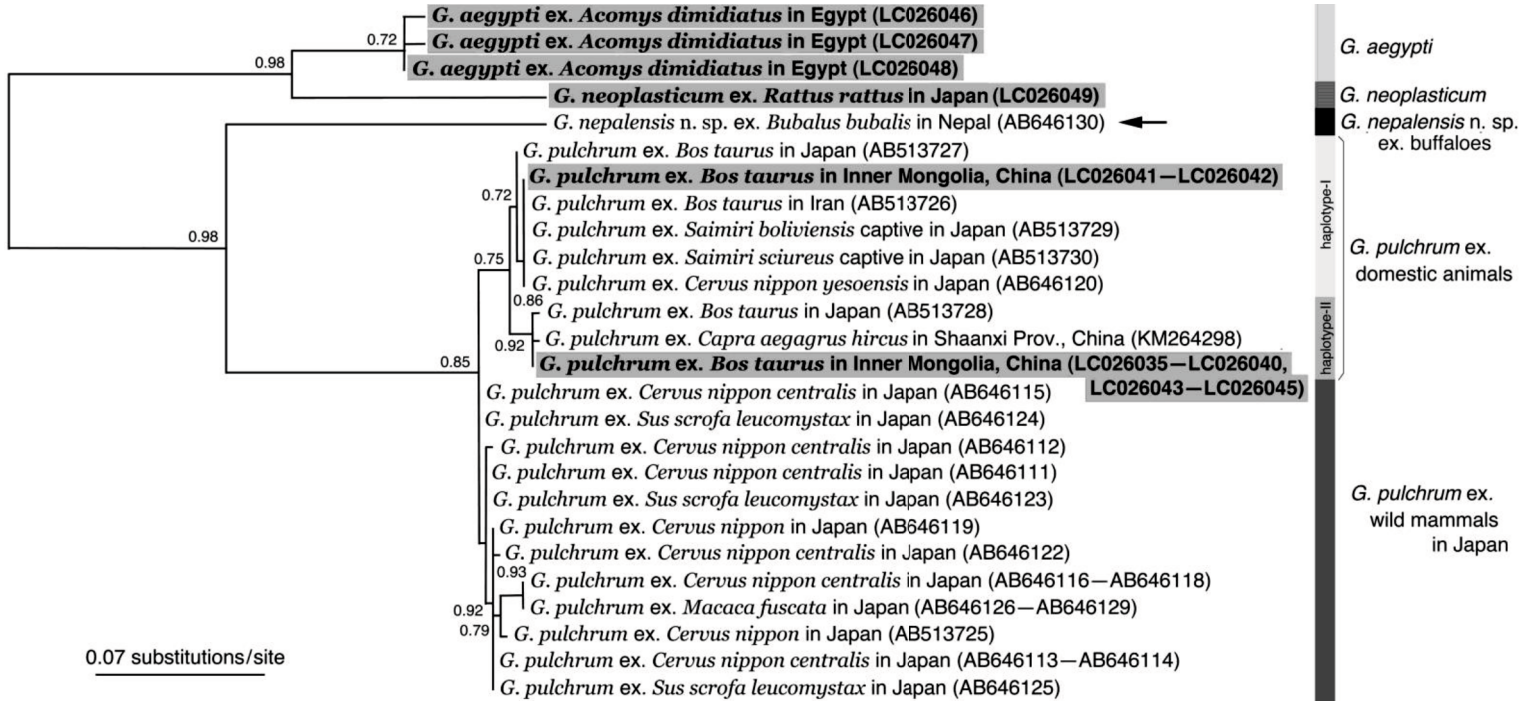

Figure 4 ML phylogenetic tree based on the *cox-1* mtDNA sequence. Species names are followed by host names, and localities (DDBJ/EMBL/GenBank accession numbers in parentheses). Isolates with a gray background are newly obtained sequences, and an arrow denotes the new species erected in the present study.

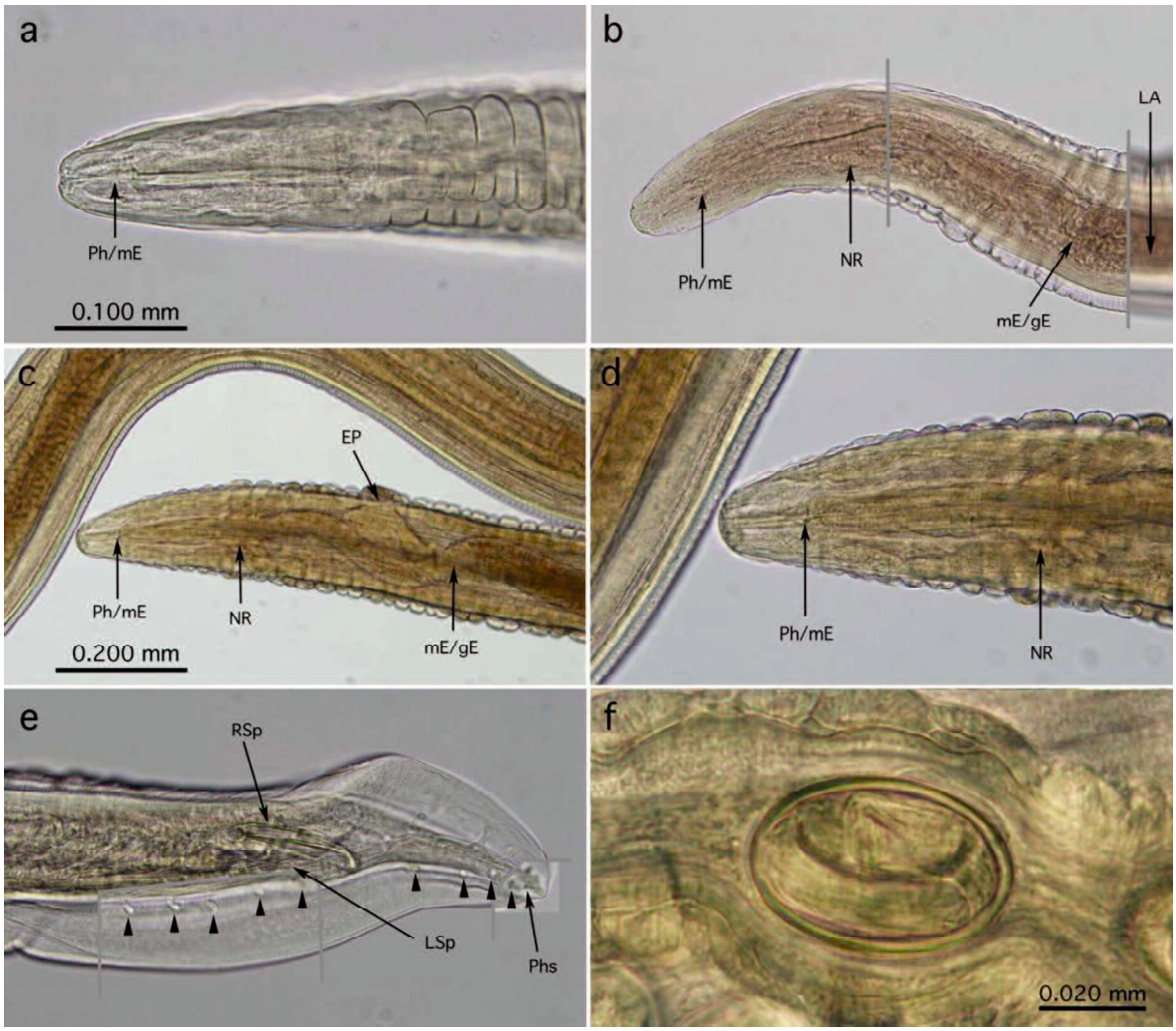

Figure 5 *Gongylonema neoplasticum* from Asian rats. (**a**, **b**) Anterior ends of male worms; (**c**, **d**) anterior end of a female worm; (**e**) posterior end of a male worm in dorsal view; and (**f**) intrauterine egg. Photographs (**b**, **e**) are composed of three segments of photographs taken at different depths, marked by gray lines. Abbreviations: EP, excretory pore; LA, lateral ala; LSp, left spicule; mE/gE, the border between muscular esophagus and glandular esophagus; NR, nerve ring; Ph/mE, the border between pharynx and muscular esophagus; Phs, phasmid; and RSp, right spicule. Arrowheads indicate caudal papillae. Photographs (**a**, **b**, **d**, and **e**) at the same magnification with scale bar in (**a**).

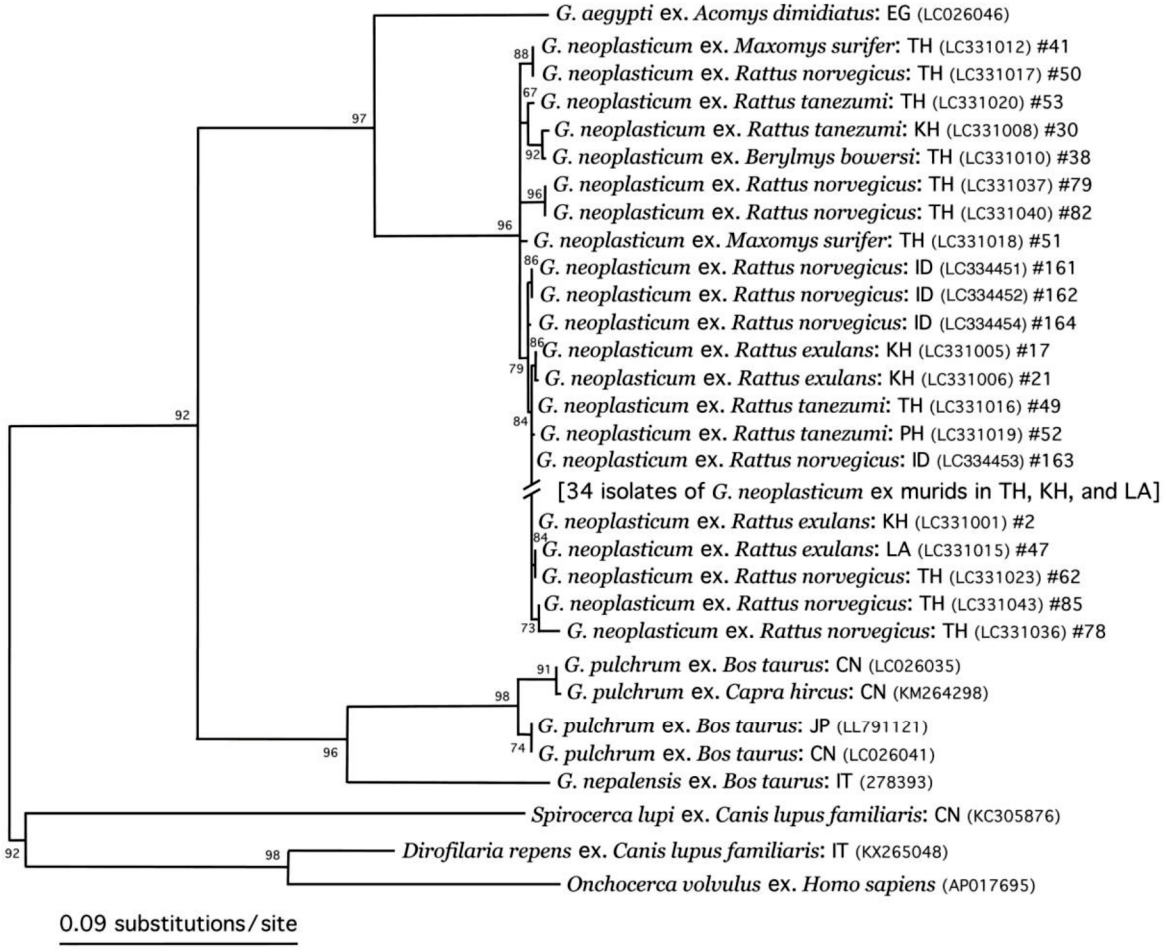

Figure 6 ML phylogenetic tree based on 818-bp long *cox-1* nucleotide sequence. All nucleotide sequences of *Gongylonema neoplasticum* shown in the figure were newly obtained in the present study, with 27 *G. neoplasticum* isolates omitted for simplification. The species name of isolates is followed by host species, country of collection, DDBJ/EMBL/GenBank accession number, and worm ID number. Abbreviations of country names: CN, China; EG, Egypt; IT, Italy; JP, Japan; KH, Cambodia; LA, Laos; PH, Philippines; and TH, Thailand.

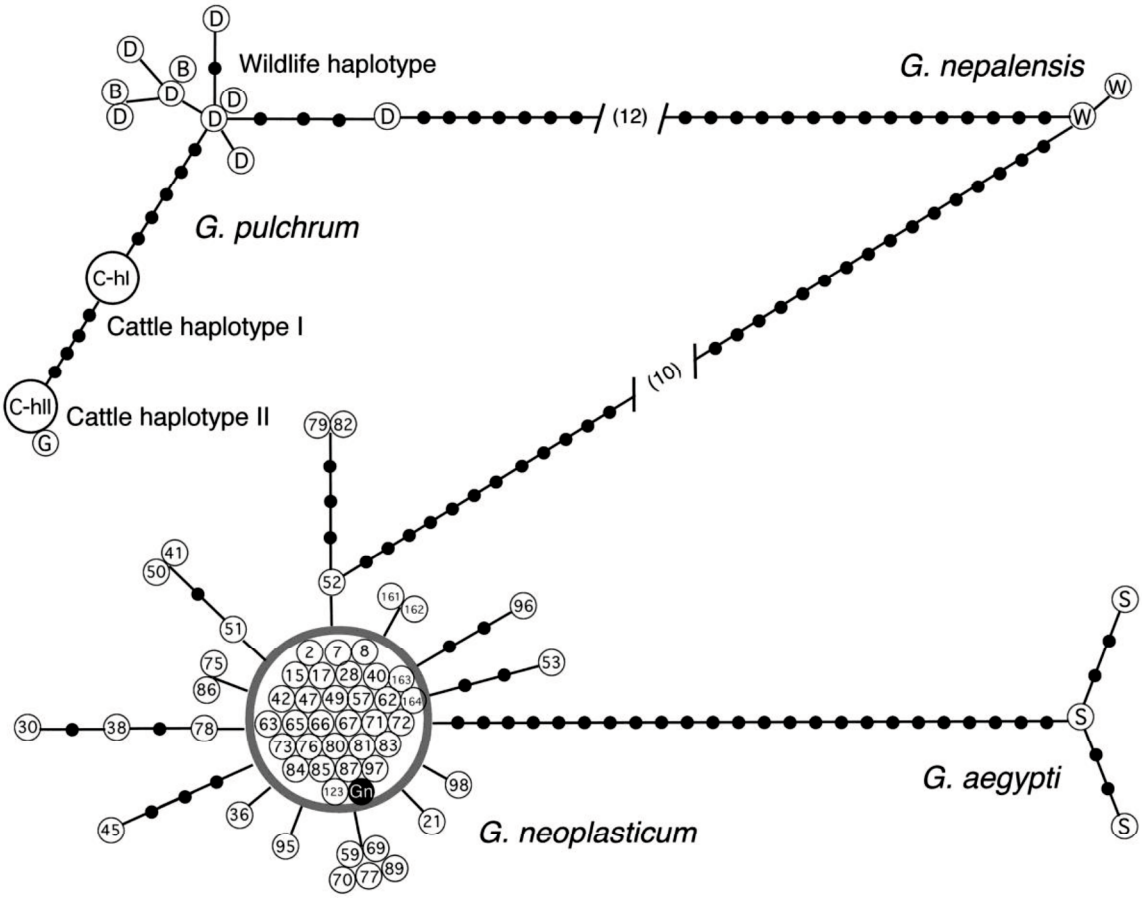

Figure 7 Relationships of *cox-1* haplotypes of *Gongylonema neoplasticum* recovered from Asian rats, based on 369-bp long nucleotide sequences. Numbers in the circles for *G. neoplasticum* indicate the worm ID number, while letters in the circles for other *Gongylonema* spp. indicate the host animal (B, wild boar; C-hI, cattle with *cox-1* haplotype I *G. pulchrum*; C-hII, cattle with *cox-1* haplotype II *G. pulchrum*; D, sika deer; G, goat; S, spiny mouse; and W, water buffalo). 'Gn' in the prominent haplotype of *G. neoplasticum* indicates the worm collected in Okinawa, Japan (DDBJ/EMBL/GenBank accession no. LC026049).

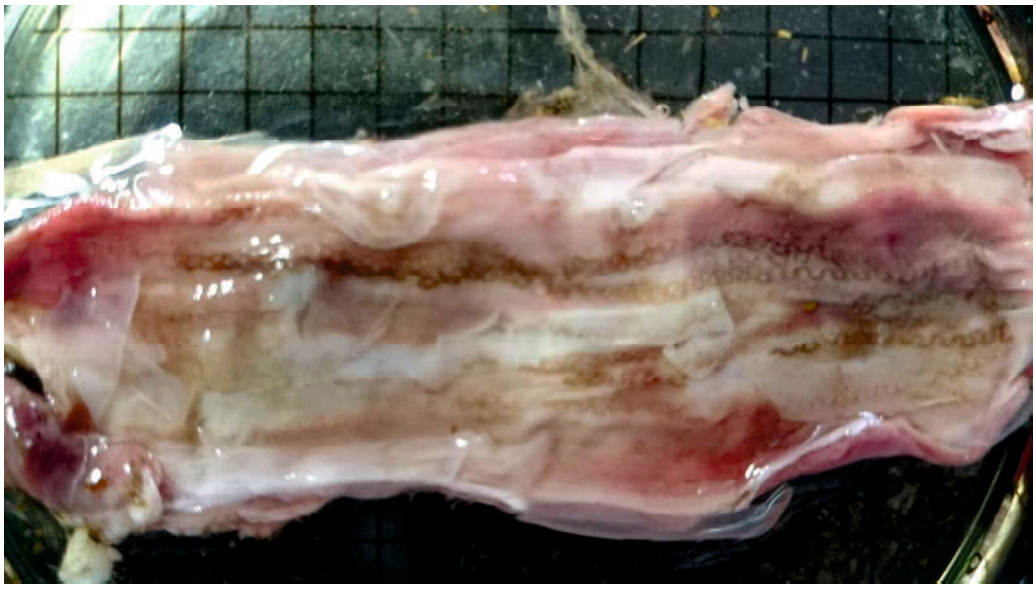

Figure 8 Gross lesions of *Gongylonema pulchrum* worm tracts in the esophageal mucosa of a feral alien Reeves's muntjac on Izu-Oshima Island, Japan. Grid lines on the bottom of the glass dish visible in the background are marked every 5 mm.

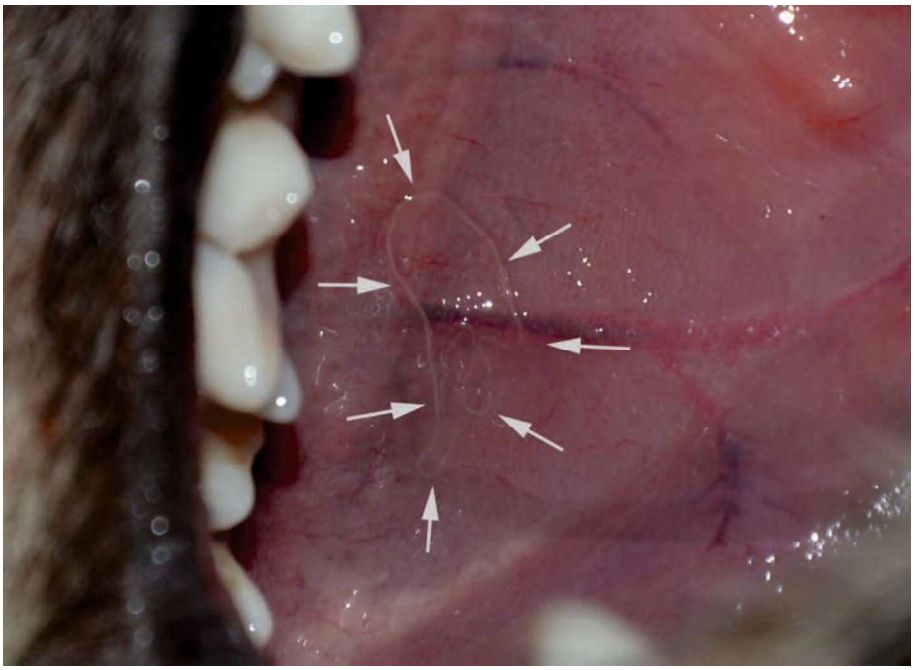

Figure 9 Gross photograph of an adult female *Gongylonema nepalensis* (arrows) parasitizing in the mucosal epithelium of the lateral back of the tongue of a red fox on Sardinia Island, Italy.

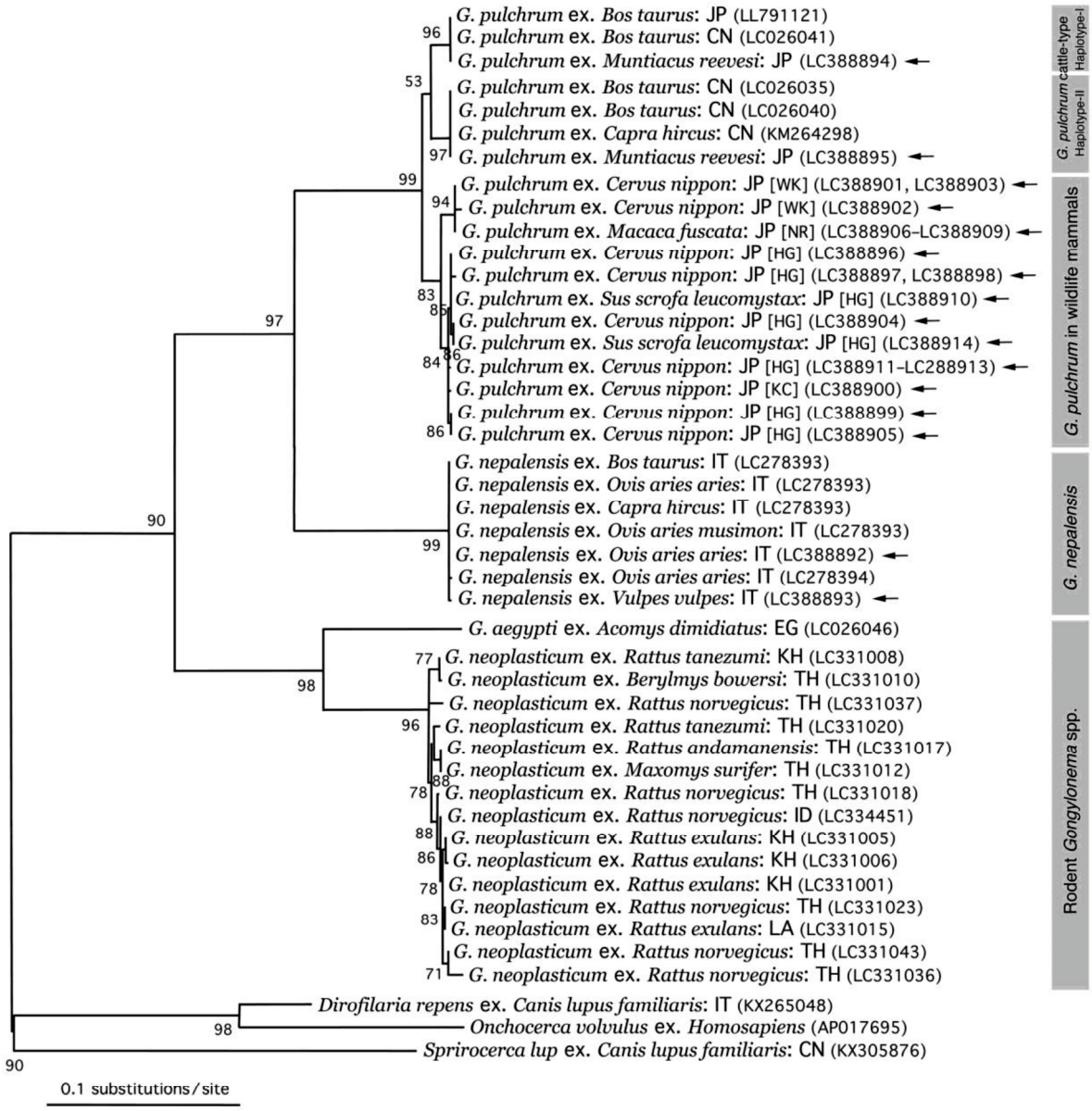

Figure 10 ML phylogenetic tree based on the cox-1 mtDNA sequences of 818-bp length. Species names are followed by host names and country names (DDBJ/EMBL/GenBank accession numbers in parentheses). New sequences denoted by arrows. Abbreviations of country names: CN, People's Republic of China; EG, Egypt; ID, Indonesia; IT, Italy; JP, Japan; KH, Cambodia; LA, Laos; and TH, Thailand. Abbreviations of prefecture names in Japan are shown in square brackets: HG, Hyogo; KC, Kochi; NR, Nara; and WK, Wakayama.