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

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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 & 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 characterisation 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 4,500 rodents: Thailand (58 rats), Cambodia (52 rats), Laos (three rats) and

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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) (5,649 bp) and the cytochrome *c* oxidase subunit 1 gene (*cox*1) (818 bp) were characterised. 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.

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 characterised 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 & Peebles, 1958; Yamaguti, 1961; Skrjabin et al., 1967; Gupta & Trivedi, 1985; Ashour & Lewis, 1986; Diouf et al., 1997; Kinsella et al., 2016). Some of the described species require the collection and characterisation of more specimens as their characterisation 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 synonymised taxa based on cross-infection experiments (Ransom & Hall, 1915; Baylis et al., 1926a, b; Schwartz & Lucker, 1931; Lucker, 1932) or meticulous morphological analyses (Schwartz & 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 & Sato, 2016 from *G. pulchrum* and understand their possible natural transmission dynamics in domestic and wild ruminants (Sato, 2009; Makouloutou et al., 2013a, b; Setsuda et al., 2016; Varcasia et al., 2017). 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 & Lewis, 1986 from the Arabian spiny mouse *Acomys dimidiatus* (Cretzschmar, 1826) on the Sinai Peninsula, Egypt, disclosing their distinctness but close relatedness (Setsuda et al., 2016). 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.

Materials and methods

Collection of parasites and morphological observation

During the last 10 years, a variety of murine rodents (approximately 4,500 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 1). 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 1). 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.

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 Illustra™ 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 20-µl volume containing a DNA polymerase, Blend Taq-Plus- (TOYOBO, Dojima

Table 1 *Gongylonema neoplasticum* worms examined in the present study

Host rodent speceis	Thailand[a]	Cambodia[a]	Laos[a]	Philippines[a]	Indonesia[b]	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[b], 17 worms were collected from four rats; nine and four worms from two rats were used for morphological and molecular genetic analyses

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 Clone™; 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′-TCC 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.

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 & Henry, 1911 (Spirurida: Onchocercidae; GenBank: KX265048), and *Onchocerca volvulus* (Leuckart, 1893) (Spirurida: Onchocercidae; GenBank: LP017695) 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 & 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 & Gascuel, 2006).

*cox*1 *haplotype analysis*

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

Results

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 1). 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

Table 2 Comparative data (measurements in mm) of *Gongylonema neoplasticum* specimens collected from murids and the European rabbit

Host	*Rattus* spp. (*R. norvegicus*, *R. exulans*, *R. tanezumi*), *Maxomys surifer*	*R. norvegicus*	*Rattus* spp. (*R. norvegicus*, *R. rattus*), *Mus musculus*	*Rattus norvegicus*? (Exp. "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	(n = 6)	(n = 6)	(n = ?)	(n = 10)	(n = 10)
Body length (BL)	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)
Pharynx length	0.029–0.061 (0.044)	0.053–0.063 (0.058)	–	0.040–0.070 (0.049)	0.028–0.039 (0.034)
Oesophagus length	1.38–2.40 (1.91)	2.99–3.50 (3.27)	2.8	2.17–3.09 (2.72)	–
Muscular oesophagus	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 oesophagus	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 length	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 BL (%)	5.3–11.2 (7.7)	4.5–5.5 (5.1)	–	–	–
Right spicule length	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 length	0.048–0.059 (0.054)	0.034–0.064 (0.054)	–	present	0.046–0.082 (0.062)
Female	(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)
Pharynx length	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)
Oesophagus length	3.34–6.14 (4.88)	6.40–7.38 (6.79)	7	–	–
Relative length to BL (%)	8.47–18.61 (11.49)	8.85–11.42 (10.42)	–	–	–
Muscular oesophagus	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 oesophagus	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	5.0–10.0 (4.80)	5.2–9.6 (5.6)
Relative length to BL (%)	9.5–11.8 (10.8)	9.8–11.4 (10.8)	10.0–12.5	10.0–12.5	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)
Egg 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 extremity. [b]From the posterior extremity

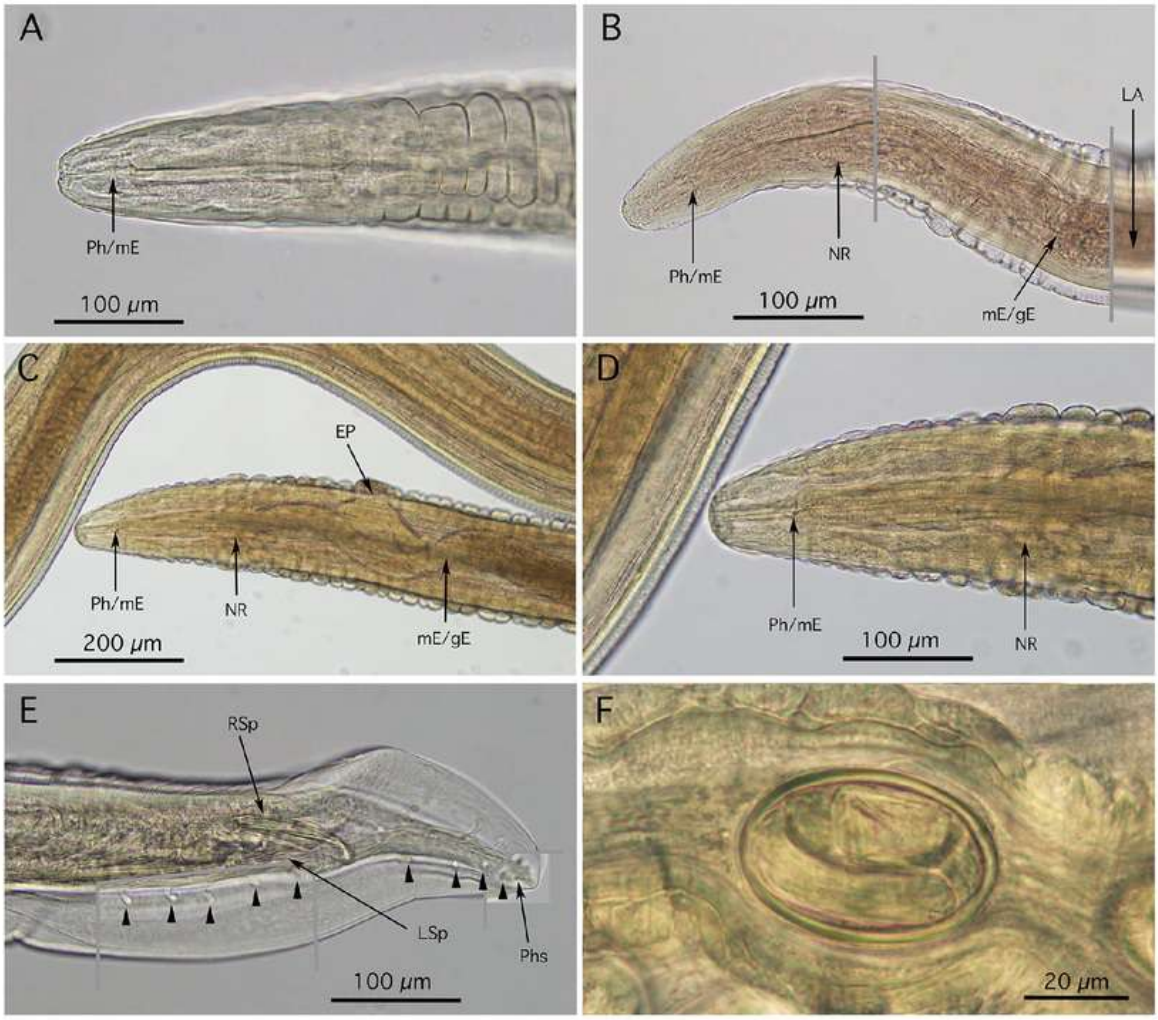

Fig. 1 *Gongylonema neoplasticum* from Asian rats. A, B, Male, anterior extremity; C, D, Female, anterior extremity; E, Male, posterior extremity, dorsal view; F, intrauterine egg. Photographs B and E are composed of three segments of photographs taken at different depths, marked by gray lines. *Abbreviations*: EP, excretory pore; LA, lateral ala; LSp, left spicule; mE/gE, the border between muscular oesophagus and glandular oesophagus; NR, nerve-ring; Ph/mE, the border between pharynx and muscular oesophagus; Phs, phasmid; RSp, right spicule. Arrowheads indicate caudal papillae

smaller sizes of male worms; see Table 2) and differently developed cuticular bosses in the anterior part of the body (poor in male worms and well developed in female worms; see Fig. 1). Mouth opening was connected to the short pharynx, then followed by the muscular and glandular oesophagi, and intestine. Male worms with asymmetric caudal alae had eight pairs of caudal papillae (four pre-cloacal 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. 1E). Male worms possessed a long left and a short right spicule (Fig. 1E). Left spicules were fine thread-like with round distal ends, whereas right spicules and gubernacula were squat. Measurements of the collected worms were well coincident with those of *G. neoplasticum* recorded in earlier studies (Table 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 3); 5,649 bp in length from near the 5′-terminus of 18S to 28S rDNA was comprised of 1,814 bp long partial 18S rDNA, 540 bp long ITS1, 158 bp long 5.8S rDNA, 478 bp long ITS2, and 2,659 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; Setsuda et al., 2016). The few nucleotide substitutions observed were located at positions 437, 579, 814 and 1,019 of the 28S rDNA (Table 4).

*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 5), 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. 2). To define

Table 3 *Gongylonema neoplasticum* worms examined for the rDNA nucleotide sequences, Worms reactive to PCR amplification of rDNA nucleotide fragments are shown by worm ID number. Specimens with numbers in bold showed a few nucleotide substitutions in the 28S rDNA (see Table 4)

Host rodent species	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**		

Table 4 Nucleotide variations in the 28S rDNA of *Gongylonema neoplasticum* of different origins. Worms with long rDNA nucleotide sequences successfully amplified by PCR are shown

Worm ID	DDBJ/ EMBL/ GenBank ID	Host	Locality	28S rDNA[a]			
				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]Relative nucleotide position to the 28S rDNA of a Japanese isolate of *G. neoplasticum* (DDBJ/EMBL/GenBank: LC026032)

Table 5 *Gongylonema neoplasticum* worms examined for the *cox*1 mtDNA nucleotide sequences

Host rodent species	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				

the molecular genetic relationship with a specimen of *G. neoplasticum* from the black rat in Okinawa, Japan (DDBJ/EMBL/GenBank: LC026049; Setsuda et al., 2016), 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 analysed by the HapStar network illustration (Fig. 3). 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 analysed 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. 3), 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.

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. 1), in addition to specimen measurements (Table 2). 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 & Ditlevsen, 1914; Yokogawa, 1925; Kruidenier & Peebles, 1958; Skrjabin et al., 1967; Singh & Cheong, 1971; Yap et al., 1977; Leong et al., 1979; Krishnasamy et al., 1980; Jueco & Zabala, 1990; Hasegawa & Syafruddin, 1995; Eira et al., 2006; Syed-Arnex & Mohd Zain, 2006; Paramasvaran et al., 2009; Dewi, 2011; Chaisiri et al.,

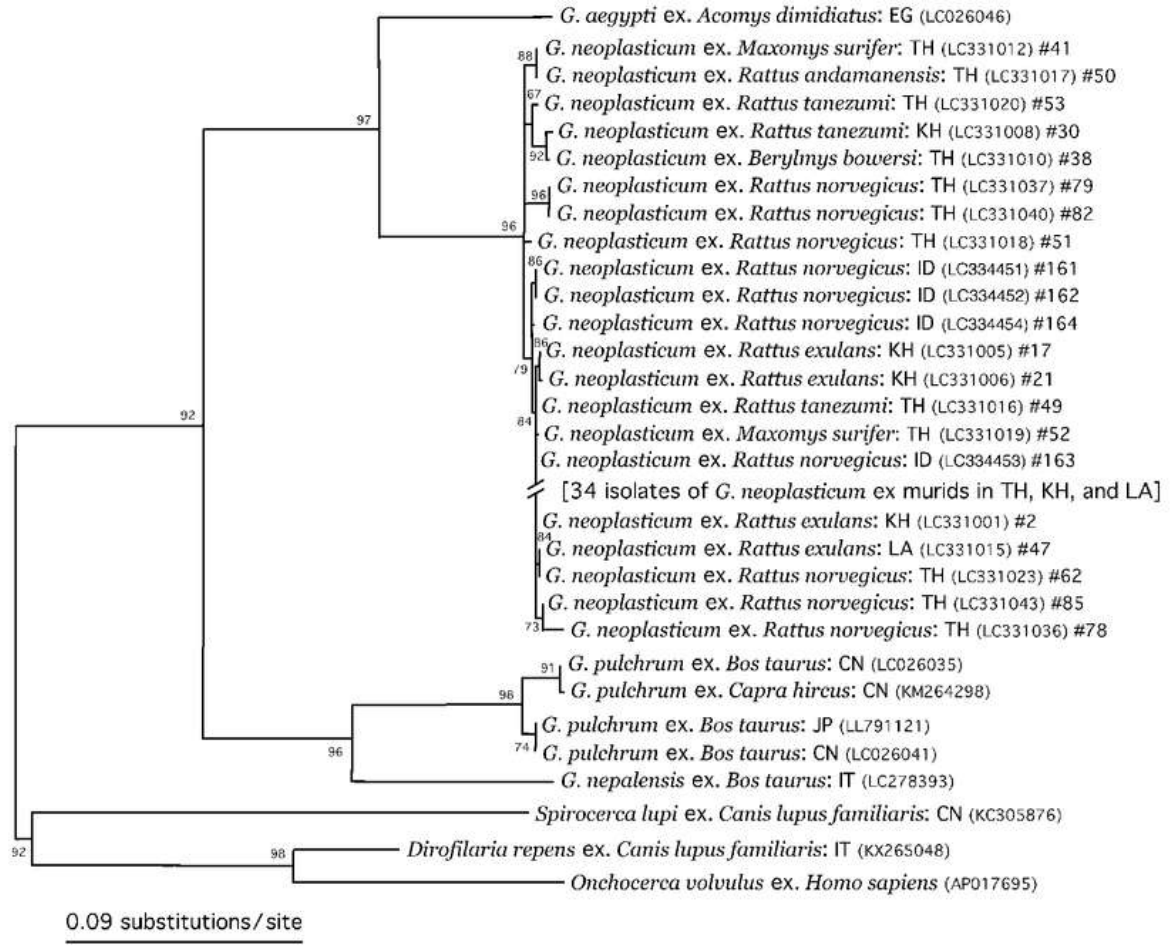

Fig. 2 Maximum Likelihood phylogenetic tree based on 818 bp long *cox*1 nucleotide sequences. All nucleotide sequences of *Gongylonema neoplasticum* shown in the figure are newly generated in the present study, with 27 isolates omitted for simplification. The species name of isolates is followed by host species, country of collection, DDBJ/EMBL/GenBank accession number, and worm ID number. *Abbreviations*: CN, China; EG, Egypt; IT, Italy; JP, Japan; KH, Cambodia; LA, Laos; PH, Philippines; and TH, Thailand

2012; Paramasvaran et al., 2012; Dewi & 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 characterise 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

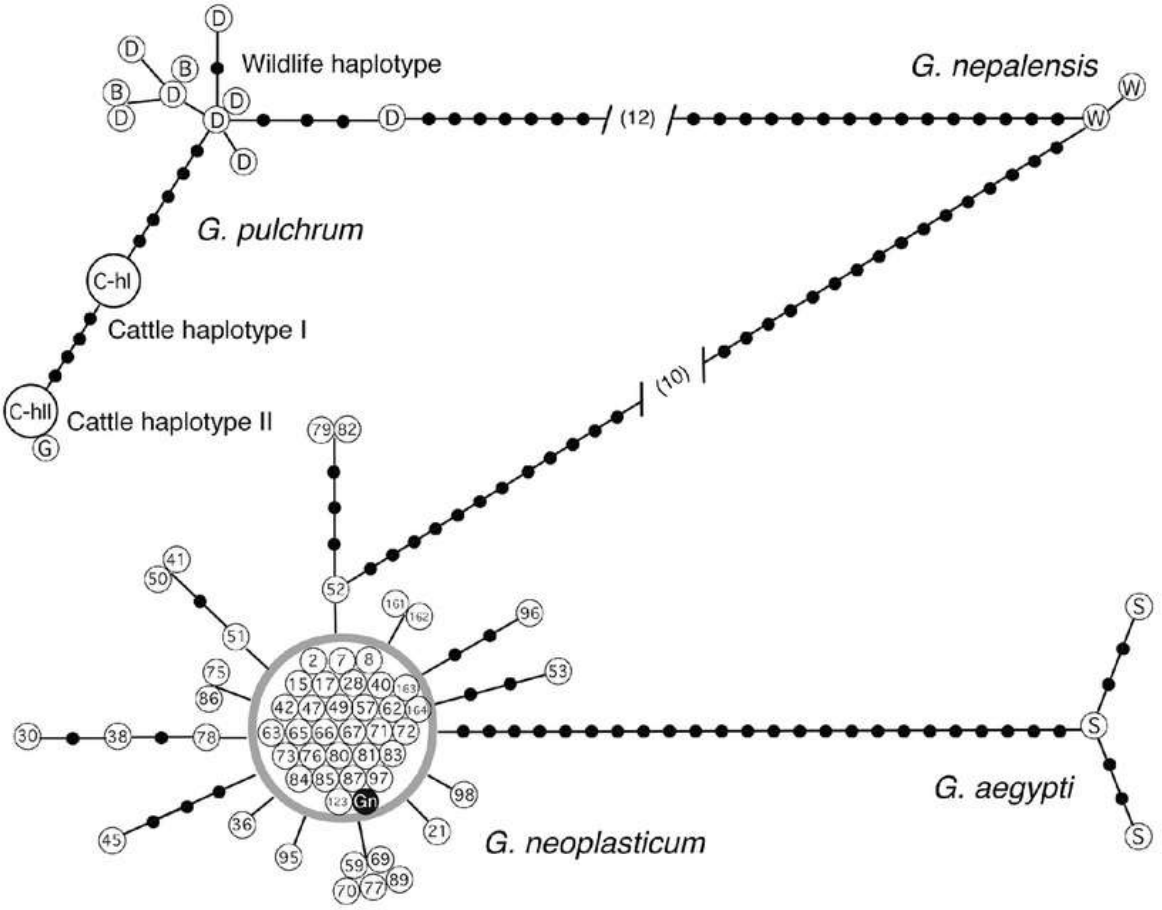

Fig. 3 Relationships of *cox*1 haplotypes of *Gongylonema neoplasticum* recovered from Asian rats, based on 369 bp long nucleotide sequences. Numbers in the circles for *G. neoplasticum* indicate the worm ID number, while letters in the circles for other *Gongylonema* spp. indicate the host animal (B, 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; W, water buffalo). 'Gn' in the prominent haplotype of *G. neoplasticum* indicates the worm collected in Okinawa, Japan (DDBJ/EMBL/GenBank: LC026049)

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 5,649 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; Setsuda et al., 2016). 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 our previous study

(Setsuda et al., 2016), we do not repeat that discussion here.

Makouloutou et al. (2013a) reported a great variety of *cox*1 gene 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; Setsuda et al., 2016). 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. 3). 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 analysed 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 (Setsuda et al., 2016). 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 & 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 analysed for its genetic uniqueness. As mentioned earlier, more than a dozen rodent *Gongylonema* spp. have been recorded to date. The molecular characterisation of each species should facilitate the phenotypical characterisation which often shows variation. Such efforts may detect substantial specific diversities of rodent *Gongylonema* spp., as previously communicated by Kinsella et al. (2016).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed.

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

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