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Chabaudstrongylus ninhae (Trichostrongylidae: Cooperiinae) and *Oesophagostomum muntiacum* (Chabertiidae: Oesophagostominae) in feral alien Reeves's muntjacs on Izu-Oshima Island, Tokyo, Japan

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Abstract

The naturalization of alien Reeves's muntjacs (*Muntiacus reevesi*) on Izu-Oshima Island, Tokyo, Japan, has proceeded intensively over the last five decades. To clarify whether the gastrointestinal helminths of these animals were brought from their original endemic area or were newly acquired in Japan, 32 Reeves's muntjacs trapped on the island were parasitologically examined. In addition to *Gongylonema pulchrum* in the oesophagus (34.4% prevalence), *Chabaudstrongylus ninhae* (Dróżdż, 1967) (Trichostrongylidae: Cooperiinae) and *Oesophagostomum muntiacum* Jian, 1989 (Chabertiidae: Oesophagostominae) were prevalent in the small (28.1%) and large (46.9%) intestines, respectively. For the first time, these trichostrongylid or chabertiid worms were genetically characterized based on partial nucleotide sequences of the nuclear ribosomal RNA gene (rDNA) and mitochondrial DNA cytochrome *c* oxidase subunit 1 gene (*cox-1*), and the phylogenetic relationships with other members of their family were explored. Since these two intestinal nematode species are inherent in muntjacs, this study demonstrates a new distribution of exotic helminth species in Japan in accordance with the naturalization of alien mammalian hosts. The molecular genetic data collected here could assist the taxonomic assessment of morphological variants in different *Muntiacus* spp. and/or of different geographical origins. Furthermore, our data may help to define the phylogenetic relationships among such isolates.

Introduction

Members of the genus *Muntiacus* Rafinesque, 1815, currently classified into 11 species with more than a dozen subspecies, are small-sized cervid species originally distributed in India, South-East Asia and southern China (Wildon & Reeder, 2005; Groves & Grubb, 2011). Reeves's muntjac, *Muntiacus reevesi* (Ogilby, 1839), is indigenous to south-eastern China and Taiwan, but naturalized in Britain and Japan, at least, after escape from an animal exhibition facility or intentional release (Chapman *et al.*, 1994; Ohdachi *et al.*, 2010). In Japan, Reeves's muntjacs are expanding their population at two localities, i.e. the southern region of the Boso Peninsula, Chiba Prefecture and Izu-Oshima Island, Tokyo, where approximately 50,000 and 17,000 muntjacs, respectively, are currently estimated to be populating the natural environment after escape from zoological facilities (Chiba Prefectural Office, 2013, 2017; Tokyo Municipal Office, 2017). The escape and naturalization of Reeves's muntjacs is thought to have happened between 1960 and 1980 on the southern Boso Peninsula, covering more than 1400 km^2, while a typhoon in the autumn of 1970 afforded their escape from Tokyo Municipal Oshima Park Zoo on the 91.1 km^2 island of Izu-Oshima. Control measures for this alien mammalian species were initiated in 2000 and 2007, respectively, and the total trapped animal numbers in 2014, 2015 and 2016 were 2160, 2187 and 2400 on the Boso Peninsula (Chiba Prefectural Office, 2017) and 1022, 1412 and 2191 on Izu-Oshima Island (Tokyo Municipal Office, 2017), respectively. However, these measures have been deemed to have had an insufficient impact on population control.

Despite the remarkable progress of the naturalization of alien Reeves's muntjacs in Japan, few parasitological investigations have been conducted. Earlier work from our laboratory reported a case of *Gongylonema pulchrum* parasitism in the oesophagus of a trapped Reeves's muntjac on Izu-Oshima Island (Makouloutou *et al.*, 2013; Setsuda *et al.*, 2018). In the present study, we examined the gastrointestinal helminths of feral alien Reeves's muntjacs trapped on Izu-Oshima Island to determine whether these animals maintain any

Table 1. Worm recovery from the intestine of feral alien Reeves's muntjacs on Izu-Oshima Island, Tokyo.

		Chabaudstrongylus ninhae			*Oesophagostomum muntiacum*		
Collection	Number of animals examined	Prevalence	Male worm[a]	Female worm[a]	Prevalence	Male worm[a]	Female worm[a]
January 2015	7	14.3% (1/7)	1	3	14.3% (1/7)	0	2
July 2015	15	40.0% (6/15)	1–20 (8.7)	0–27 (10.8)	66.7% (10/15)	0–65 (12.4)	4–73 (20.0)
October 2016	10	20.0% (2/10)	1, 3	2, 1	40.0% (4/10)	0–58 (29.0)	1–90 (45.3)
Total	**32**	**28.1% (9/32)**	**1–20 (6.3)**	**0–27 (7.9)**	**46.9% (15/32)**	**0–65 (16.0)**	**1–90 (25.5)**

[a] Worm recovery/animal, expressed as range and average in parentheses.

gastrointestinal helminths brought from their original endemic area or have newly acquired ones following their introduction to Japan.

Materials and methods

Parasite collection and morphological examination

Viscera 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 (ten animals) with the permission of the Tokyo Metropolitan Oshima Island Branch Office. All muntjacs of either sex were mature adults with body weights of 6.6–10.0 kg. Referring to the growth curve data of Asada (2014) indicating that the average body weights of male and female muntjacs naturalized on the Boso Peninsula reach 9.6 kg and 8.8 kg, respectively, at 1.5 years of age then plateau, all examined muntjacs were older than six months. Frozen viscera were transported to the Laboratory of Veterinary Parasitology, Yamaguchi University, and helminth parasites were collected according to a standard procedure (Sultan *et al.*, 2014). Briefly, organs were separated, placed in different vessels, opened longitudinally or cut into blocks and washed in tap water. Following repeated washings, the sediments were checked under a dissection microscope. Collected parasites were washed in physiological saline and preserved in either neutral-buffered 10% formalin or 70% alcohol. For scanning electron microscopy (SEM), formalin-fixed worms were processed as described previously (Tran *et al.*, 2015).

DNA extraction, polymerase chain reaction (PCR) and sequencing

Parasite DNA was individually extracted from worms according to Setsuda *et al.* (2016). PCR amplification of overlapping fragments of the nuclear ribosomal RNA gene (rDNA) was performed in a 25 µl volume using different primer combinations as previously described (Makouloutou *et al.*, 2013). The mitochondrial DNA cytochrome *c* oxidase subunit 1 gene (*cox-1*) region of worms was amplified by primer pairs of BpCoxI-F1 (5′-TTTGGTCATCCTGAGGTTTATATT-3′) and BpCoxI-R1 (5′-ATGAAAATGTCTAACTACATAATAAGTATC-3′), or CapiCox1_F (5′-GTGGTTTTGGTAATTGAATGGTA-3′) and BpCoxI-R1, according to Sultan *et al.* (2014). The purification of PCR products using a commercial kit and subsequent nucleotide sequencing, including that of rDNA fragments containing the internal transcribed spacer (ITS) regions, were conducted as described in Setsuda *et al.* (2016). The obtained sequences were assembled manually 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 LC415111–LC415114.

Phylogenetic analysis

Fragments of the newly obtained rDNA sequences and partial *cox-1* sequences were submitted to the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website to identify highly similar nucleotide sequences. Phylogenetic trees were constructed for the newly obtained sequences in the present study and those retrieved from the DDBJ/EMBL/GenBank databases. The accession numbers of the sequences analysed in the present study are given in the figures showing phylogenetic trees. 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 670 characters, of which 211 were variable, for trichostrongylid nematodes, or 679 characters, of which 200 were variable, for chabertiid nematodes. The probability of inferred branches was assessed by the approximate likelihood ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova & Gascuel, 2006).

Results

Prevalence of worms

In addition to *G. pulchrum* in the oesophageal mucosa of 11 (34.4%) Reeves's muntjacs, trichostrongylid and chabertiid nematodes were collected from the small and large intestines, respectively. Although the prevalence and intensity were rather varied at different collection times (table 1), 28.1% and 46.9% of a total of 32 examined muntjacs were parasitized with these nematodes, with an intensity of 1–47 (average 14.2) and 1–148 (average 41.5) worms, respectively.

Chabaudstrongylus ninhae *in the small intestine*

Trichostrongylid nematodes collected from the small intestine had an oval cephalic vesicle, caudal bursa with a long dorsal ray deeply divided, didelphic uterus and synlophes, of which the

Table 2. Comparison of measurements of *Chabaudstrongylus* spp. from muntjacs and chevrotains (in mm).[a]

Species	*Chabaudstrongylus ninhae*	*Chabaudstrongylus ninhae* (syn. *Cooperia ninhae*)[b]	*Chabaudstrongylus dubosti* (syn. *Cooperia dubosti*)[b]
Host	*Muntiacus reevesi*	*Muntiacus muntjak*	*Hyemoschus aquaticus*
Locality	Izu-Oshima Island, Tokyo, Japan	Tuyen-Quang Province, Northern Vietnam	Makokou, Gabon
Reference	Present study	Dróżdż (1967)	Durette-Desset & Chabaud (1974)
Male worm			
Number of worms examined	6	6	Many
Body length	4.94–5.91 (5.40)	5.5–6.0	6.5
Max. body width	0.087–0.092 (0.090)	0.092–0.101	0.140
Length of cephalic vesicle	0.029–0.041 (0.035)	(0.022–0.025 in diameter)	0.192
Width of cephalic vesicle	0.033–0.041 (0.038)		0.050
Length of oesophagus	0.272–0.374 (0.343)	0.344–0.351	0.390
Max. width of oesophagus	0.027–0.033 (0.031)	0.032–0.037	—
Nerve ring[c]	0.122–0.152 (0.135)	0.136–0.143	0.290
Excretory pore[c]	—	—	0.415
Spicule length	0.129–0.140 (0.134)	0.153–0.162	0.180
Spicule width	0.017–0.023 (0.021)	0.021–0.027	—
Female worm			
Number of worms examined	6	12	many
Body length	6.31–7.49 (6.86)	7.0–8.3	8.8
Max. body width	0.091–0.110 (0.096)	0.106–0.118	0.130
Length of cephalic vesicle	0.039–0.076 (0.058)	—	—
Width of cephalic vesicle	0.036–0.049 (0.043)	—	—
Length of oesophagus	0.331–0.382 (0.359)	—	0.380
Max. width of oesophagus	0.028–0.038 (0.035)	—	—
Nerve ring[c]	0.140–0.151 (0.147)	—	0.270
Excretory pore[c]	—	—	0.375
Vulva[d]	1.42–1.66 (1.53)	1.8–1.9	2.25
Length of ovejector between sphincters	0.242–0.342 (0.290)	0.339–0.445	0.555
Tail length	0.131–0.176 (0.154)	0.155	0.200
Egg size	0.063–0.071 (0.067) x 0.030–0.039 (0.033)	0.071–0.074 x 0.034–0.037	0.068 x 0.038

[a] Values are expressed in range and average in parentheses, if available. '—' denotes no data.
[b] Synonymized by Durette-Desset & Denké (1978).
[c] From the anterior end.
[d] From the posterior end.

lateral ridges were smaller than the median ridges. These morphological characters were consistent with the definition of the subfamily Cooperiinae (Skrjabin et Schultz, 1937) of the family Trichostrongylidae (Leiper, 1908), according to Lichtenfels (1980). Although the present specimens had a simple cephalic vesicle, other characters such as a short ovejector, less than 0.50 mm, and a long dorsal ray divided in the posterior third matched exactly with the definition of the genus *Chabaudstrongylus* Durette-Desset et Denké, 1978. In this genus, *C. ninhae* (Dróżdż, 1967) from an Indian muntjac (*Muntiacus muntjak*) in northern Vietnam and *C. dubosti* (Durette-Desset et Chabaud, 1974) Durette-Desset et Denké, 1978, from a water chevrotain (*Hyemoschus aquaticus*) in Gabon have been described (Dróżdż, 1967; Durette-Desset & Chabaud, 1974; Durette-Desset & Denké, 1978), and almost all the morphological characters of the present specimens were identical to those of the former species (table 2). A brief description of the present *C. ninhae* specimens is as follows.

Small-sized worms, 5–8 mm in length, with slight sexual dimorphism. Anterior end of both sexes with simple cephalic vesicle

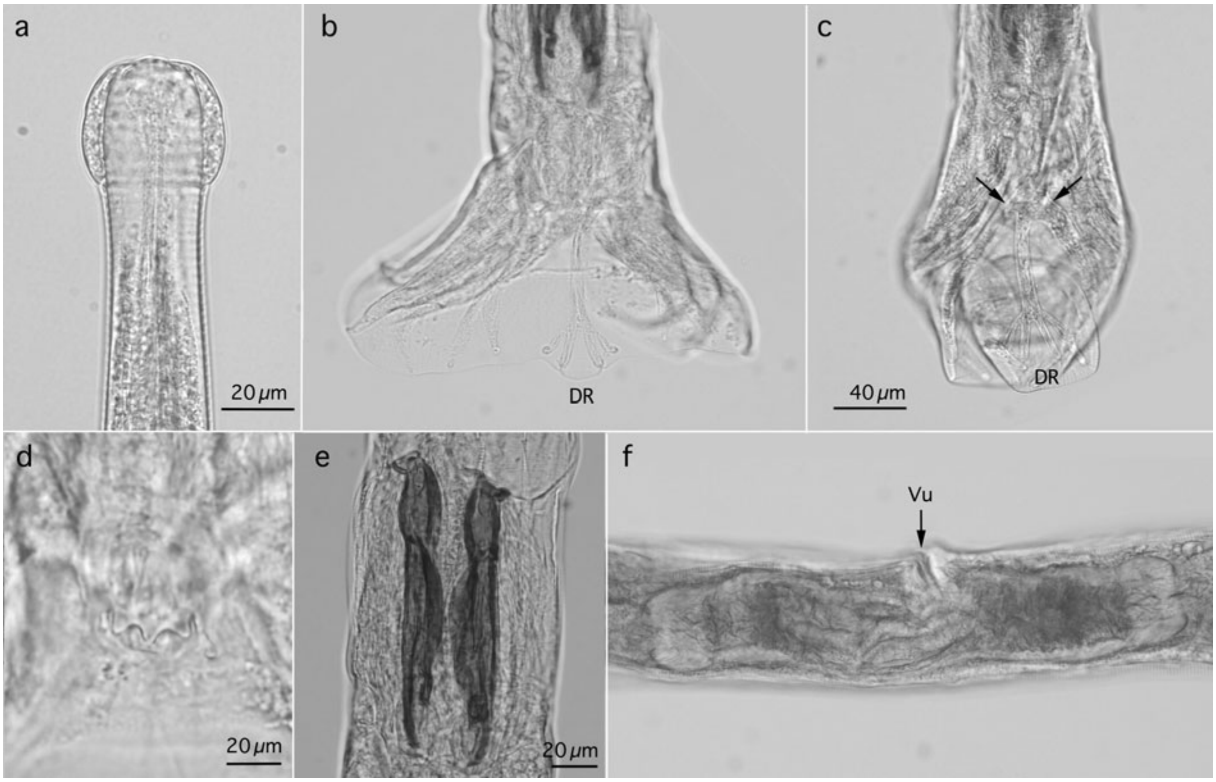

Fig. 1. Morphology of *Chabaudstrongylus ninhae* from feral alien Reeves's muntjacs. (a) Anterior end of male worm with a simple cephalic vesicle; (b, c) Ventral and dorsal views, respectively, of caudal end of male worm with a branched dorsal ray (DR) in the symmetrical copulatory bursa. Arrows indicate base of externodorsal ray; (d) Genital cone of male worm; (e) Alate, trifurcated spicules of male worm; (f) Ovejector between sphincters. Vu, vulva. Magnifications of photographs (b), (c) and (f) are identical and scale bar is shown in (c).

(fig. 1a). Synlophe well developed over entire worm length, with its pattern identical to that of *C. dubosti* (see Fig. 1E of Durette-Desset & Chabaud, 1974 for *C. dubosti*). Copulatory bursa developed symmetrically, with the dorsal ray branched in the posterior third (fig. 1b). Externodorsal ray originated from the dorsal ray as illustrated in Fig. 1G of Durette-Desset & Chabaud (1974) for *C. dubosti* (fig. 1c). Peripheral ends of all rays extending to the edge of the bursa. Genital cone was characteristic structure, as shown in fig. 1d, which showed close resemblance to that of *Chabaudstrongylus* sp. illustrated by Gibbons and Khalil (1983). Two alate spicules golden brown in colour and equal in size and shape with ventral and dorsal processes at the peripheral end (fig. 1e). Vulva situated near the posterior fourth of female worms, transverse slit, connected directly to the ovejector (fig. 1f). Except for the bifurcation of internal peripheral branches of dorsal rays reported in the original description, the present specimens were identical in morphology to *C. ninhae* described by Dróżdż (1967).

The present specimens and *C. dubosti* differed morphologically regarding the shape of the cephalic vesicle (simple vs. elongated), position of the nerve ring (at mid-oesophagus vs. almost at posterior fourth of oesophagus) and shape and size of spicules. However, these two species appeared to be closely related, particularly with regard to an almost identical arrangement and size of rays in the copulatory bursa.

Oesophagostomum muntiacum *in the large intestine*

Chabertiid nematodes collected from the caecum and colon had a small and shallow buccal capsule, transverse cervical groove at the level of the excretory pore and externodorsal rays arising from a common stem with the dorsal ray. These morphological characters were consistent with the definition of the subfamily Oesophagostominae (Railliet, 1916) of the family Chabertiidae (Popova, 1952), according to Durette-Desset (1983). Morphological characters of the present specimens such as an incomplete transverse cervical groove at the level of the excretory pore, external corona radiata with ten elements, internal corona radiata with 20 elements and short spicules less than 0.5 mm matched exactly with the definition of the genus *Oesophagostomum* Railliet et Henry, 1913. In this genus, *Oesophagostomum* (*Hysteracrum*) sp. (Yen, 1973) from northern red muntjacs (*Muntiacus vaginalis*) in southern China, *O. labiatum* (Dróżdż, 1973) from Indian muntjacs in northern Vietnam and *O. muntiacum* Jian, 1989 from Reeves's muntjacs in central China have been described (Dróżdż, 1973; Yen, 1973; Jian, 1989), and mostly the morphological characters of the present specimens were identical to those of these species. Furthermore, *Neorhabditostomum yeni* Gibbons et Kan, 1987, resembling the three aforementioned species in morphology, was described from Indian muntjacs in Malaysia (Gibbons & Kan, 1987). The genus *Neorhabditostomum* Gibbons et Kan, 1987, of the subfamily Oesophagostominae, was proposed based on a cephalic extremity with a single leaf crown of ten elements, and six internal labial papillae, transverse cervical groove completely encircling the body and unsegmented, and elongate oesophageal funnel (Gibbons & Kan, 1987). Morphologically, the present specimens were almost identical to *O. muntiacum*, different from *O. labiatum* and *N. yeni.* A morphometric comparison of the present specimens with hitherto known *Oesophagostomum* spp. from muntjacs is shown in table 3.

Table 3. Comparison of measurements of *Oesophagostomum* spp. from muntjacs (in mm).[a]

Original name of species	*Oesophagostomum muntiacum*	*Oesophagostomum muntiacum*	*Oesophagostomum (Hysteracrum)* sp.	*Oesophagostomum labiatum*	*Neorhabditostomum yeni*
Host	Muntiacus reevesi	*Muntiacus reevesi*	*Muntiacus vaginalis*	*Muntiacus muntjak*	*Muntiacus muntjak*
Locality	Izu-Oshima Island, Tokyo Japan	Shaanxi Province, China	Yunnan Province, China	Tuyen-Quang Province, Northern Vietnam	Sabah State, Malaysia
Reference	Present study	Jian (1989)	Yen (1973)	Dróżdż (1973)	Gibbons & Kan (1987)
Male worm					
Number of worms examined	6	?	?	10	3
Body length	3.19–3.73 (3.50)	3.78–4.26 (4.03)	3.04–4.30	7.5–8.5	3.35–3.75
Max. body width	0.077–0.109 (0.094)	0.150–0.190 (0.168)	—	0.125–0.154	0.118–0.125
Length of cephalic vesicle	0.107–0.126 (0.119)	0.110–0.123 (0.114)	0.120–0.129	—	0.103–0.125
Width of cephalic vesicle	0.033–0.039 (0.036)	0.100–0.108 (0.105)	—	—	—
Length of oesophagus	0.172–0.196 (0.182)	0.180–0.200 (0.195)	0.192–0.216	—	0.194–0.211
Max. width of oesophagus	0.029–0.050 (0.044)	—	—	—	0.062–0.065
Nerve ring[b]	0.082–0.120 (0.092)	0.105–0.110 (0.109)	—	—	0.100–0.124
Excretory pore[b]	0.107–0.126 (0.119)	0.410–0.440 (0.424)	—	—	0.097–0.113
Spicule length	0.379–0.447 (0.420)	0.410–0.440 (0.424)	0.347–0.432	0.499–0.559	0.454–0.557
Gubernaculum length	0.044	0.031–0.038 (0.033)	0.046	—	0.043–0.060
Female worm					
Number of worms examined	6	?	?	28	3
Body length	3.96–4.58 (4.23)	4.28–4.54 (4.52)	3.28–4.40	8.5–9.0	3.66–4.12
Max. body width	0.097–0.122 (0.110)	0.080–0.100 (0.094)	—	0.211–0.244	0.118–0.142
Length of cephalic vesicle	0.112–0.131 (0.118)	0.105–0.118 (0.113)	—	—	0.112–0.122
Width of cephalic vesicle	0.034–0.041 (0.039)	0.065–0.100 (0.076)	—	—	—
Length of oesophagus	0.180–0.190 (0.186)	0.190–0.200 (0.197)	0.182–0.226	0.386–0.416	0.194–0.211
Max. width of oesophagus	0.044–0.058 (0.050)	—	—	0.086–0.089	0.065–0.070
Nerve ring[b]	0.077–0.105 (0.087)	0.110–0.120 (0.116)	—	—	0.108–0.132
Excretory pore[b]	0.112–0.131 (0.118)	—	—	—	0.094–0.103
Vulva[c]	0.472–0.556 (0.517)	0.500–0.550 (0.510)	0.297–0.480	0.364–0.396[d]	0.377–0.422
Tail length	0.287–0.335 (0.307)	0.290–0.330 (0.320)	0.190–0.300	0.119–0.134	0.252–0.269
Egg size	0.064–0.066 × 0.031–0.033	0.055–0.063 × 0.028–0.030	—	0.065–0.069 × 0.026–0.036	0.058–0.067 × 0.026–0.036

[a] Values are expressed in range and average in parentheses, if available. '—' denotes no available data.

[b] From the anterior end.

[c] From the posterior end.

[d] Values reported in the original article (0.245–0.262 mm from the posterior end) are apparently a mistake, and these must be the distances between the vulva and anus. By adding distance values between the anus and posterior end, estimated values are provided here.

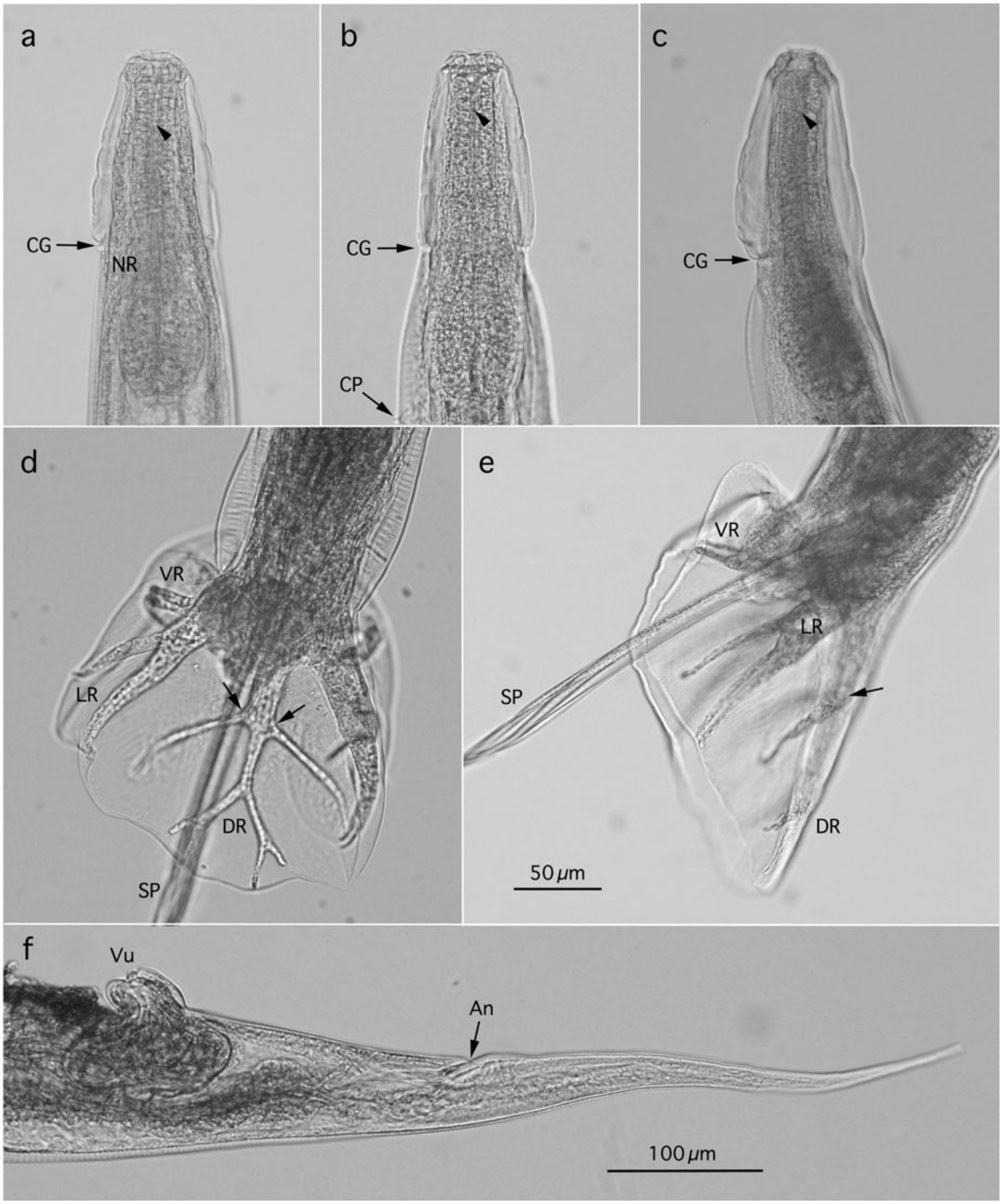

Fig. 2. Morphology of *Oesophagostomum muntiacum* from feral alien Reeves's muntjacs. (a, b) Lateral and ventral views, respectively, of anterior end of male worm with a cephalic vesicle delineated by the cervical groove (CG). Arrowhead indicates bottom of oesophageal funnel; (c) Lateral view of anterior end of female worm; (d) Dorsal view of caudal end of male worm with a branched dorsal ray (DR) in the symmetrical copulatory bursa. Arrows indicate base of externodorsal ray; (e) Lateral view of caudal end of male worm with a developed copulatory bursa supported by ventral, lateral and dorsal rays. Arrow indicates base of externodorsal ray; (f) Lateral view of caudal end of female worm. CP, cervical papilla; NR, nerve ring; LR, lateral ray; SP, spicule; VR, ventral ray; An, anus; Vu, vulva. Magnifications of photographs (a–e) are identical and scale bar is shown in (e).

A brief description of the present specimens, *O. muntiacum*, is as follows.

Small-sized worms, 3–5 mm in length, with sexual dimorphism. Anterior end of both sexes with cephalic vesicle, delineated by cervical groove, particularly on the ventral side (fig. 2a–c). Excretory pores at the level of the cervical groove and cervical papillae at the level just posterior to the end of the oesophagus. Oesophageal funnel conspicuous and nerve ring at the border between the oesophageal corpus and bulb. Head delineated from cephalic vesicle, with four sub-median papillae and two amphids (fig. 3a–c). Mouth

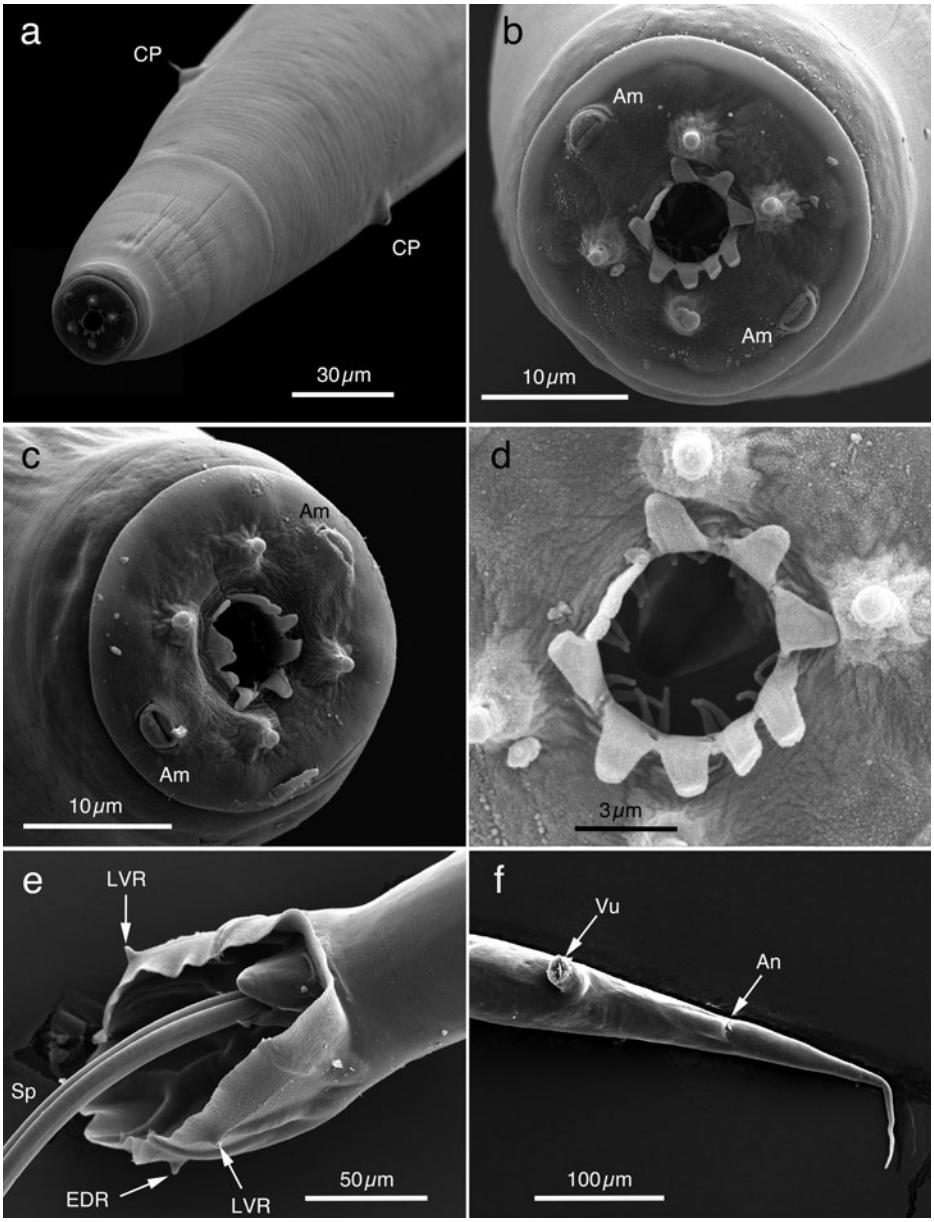

Fig. 3. SEM of *Oesophagostomum muntiacum* from feral alien Reeves's muntjacs. (a) Anterior end of male worm with cervical papillae (CP); (b) *En face* view of head of male worm shown in (a), delineated from cephalic vesicle, with four sub-median papillae and two amphids (Am). Mouth opening is surrounded by external and internal coronae radiatae. External corona radiata with ten petal-like elements and two slender elements of internal corona radiata at the base of each external corona radiata element; (c) Head of female worm, delineated from cephalic vesicle, with four sub-median papillae and two amphids (Am). Mouth opening is surrounded by ten petal-like elements of external coronae radiata; (d) Mouth opening of female worm shown in (b), surrounded by external and internal coronae radiatae; (e) Ventral view of copulatory bursa of male worm with protruded spicules (Sp). Some rays supporting the copulatory bursa do not reach the edge of the membrane (their tips are visible); (f) Ventral view of caudal portion of female worm. EDR, externodorsal ray; LVR, lateroventral ray; Vu, vulva; An, anus.

opening surrounded by external and internal coronae radiatae. External corona radiata with ten petal-like elements and two slender elements of each internal corona radiata at the base of each external corona radiata element (fig. 3d). Copulatory bursa developed symmetrically, as illustrated in Fig. 10 of Gibbons & Kan (1987), with externodorsal rays arising from a common stem with the dorsal ray (figs 2d, e and 3e). Spicules equal and slender, with a gubernaculum. Caudal portion of female worms tapering gradually with long slender tails, measuring 0.287–0.335 mm (fig. 2f). Vulval lips at 0.176–0.231 mm anterior to the anus. Intrauterine eggs oval, embryonated, with thin shells.

Neorhabditostomum yeni and *O. muntiacum* were independently described by Gibbons & Kan (1987) and Jian (1989), respectively, to identify the specimens of Yen (1973), *Oesophagostomum* (*Hysteracrum*) sp. Due to this reason, these three species resemble one another well morphologically, although some characters were characterized differently by the different authors. The *Oesophagostomum* specimens collected in the present study appeared to be identical to *O. muntiacum*, differentiated from *N. yeni* by its specific and/or generic definitions such as a cephalic extremity with a single leaf crown of ten elements without internal corona radiata, and six internal labial papillae, transverse cervical groove completely encircling the body, and longer spicules (0.454–0.557 mm vs. 0.379–0.447 mm when the body lengths of both species were almost identical) according to Gibbons & Kan (1987). Although *O. labiatum* recorded from Indian muntjacs in northern Vietnam (Dróżdż, 1973) had the same arrangement of bursal rays as *O. muntiacum*, it differed from *O. muntiacum* with respect to external corona radiata with six elements, internal corona radiata with 12 elements, cervical papillae located slightly behind the cephalic vesicle and worm dimensions (table 3).

Phylogenetic analysis

The rDNA nucleotide sequences were obtained for *C. ninhae* and *O. muntiacum* (5236-bp and 5165-bp long, respectively). The

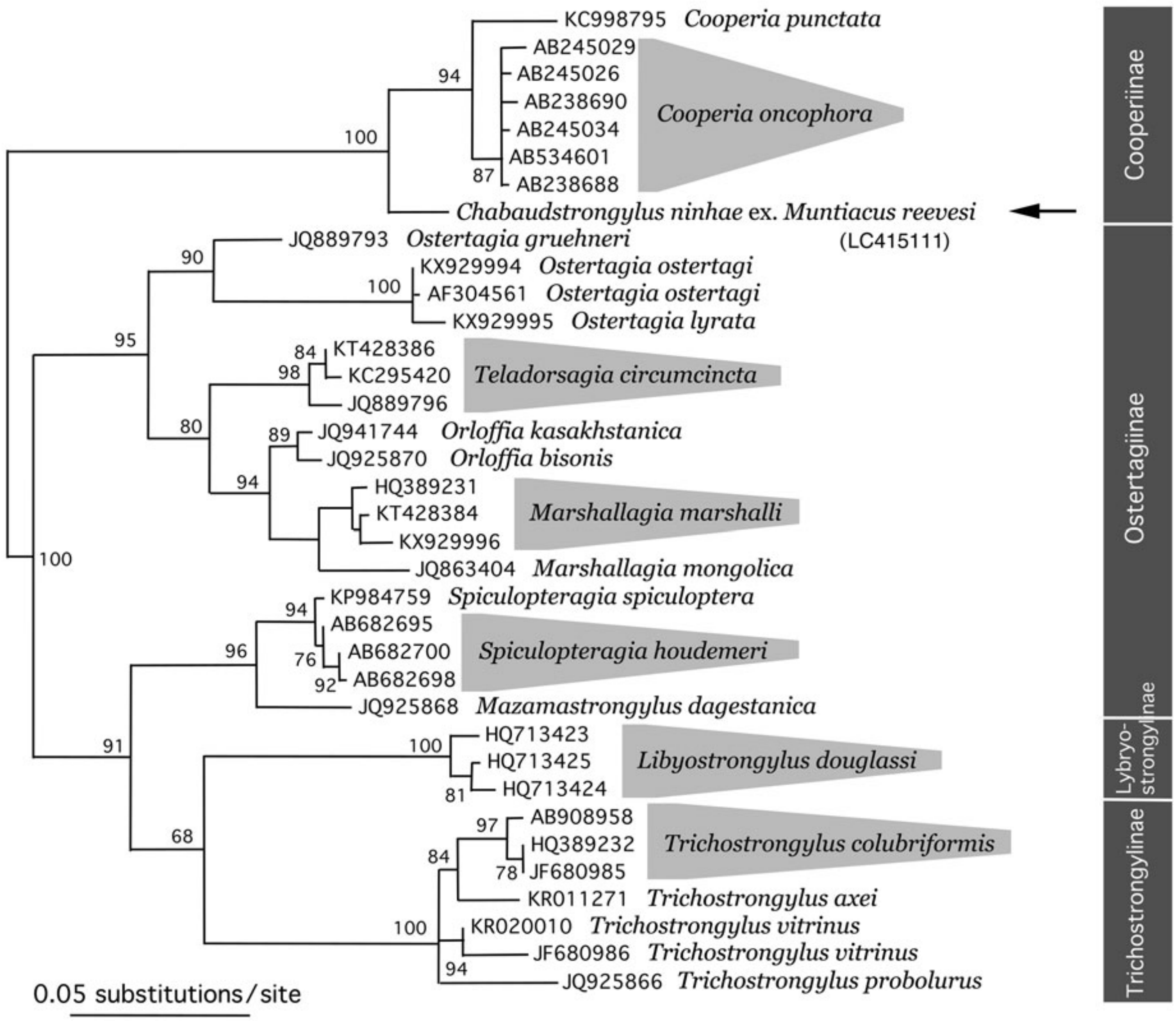

Fig. 4. ML phylogenetic tree based on the ITS regions of rDNA sequence. Representatives of different species of Trichostrongylidae are shown and they are depicted by their DDBJ/EMBL/GenBank accession numbers. The original analyses were conducted with the following additional sequences: *Cooperia oncophora* (AB238687, AB238689, AB245027, AB245028, AB245030–AB245033 and AB245035–AB245045); *Teladorsagia circumcincta* (HQ389230, JF680984, JQ889795, JQ889797, KC295419 and KX929991–KX929993); *Marshallagia marshalli* (JQ839283, KC295417 and KC295418); *Spiculopteragia houdemeri* (AB367797, AB682690–AB682694, AB682696, AB682697, AB682699, AB682701 and AB682702); *Libyostrongylus douglassi* (HQ713426–HQ713430); *Trichostrongylus colubriformis* (AB908959, AB908960, HQ389229–HQ389231, KR002110 and KR002111); *Trichostrongylus vitrinus* (KR020011); and *Trichostrongylus probolurus* (JQ925867). Arrow indicates the newly obtained rDNA nucleotide sequence of *Chabaudstrongylus ninhae* in the present study.

former sequence contained 1739-bp-long partial 18S rDNA, 414-bp-long ITS1, 144-bp-long 5.8S rDNA, 242-bp-long ITS2 and 2697-bp-long partial 28S rDNA, and the latter 1737-bp-long partial 18S rDNA, 372-bp-long ITS1, 144-bp-long 5.8S rDNA, 216-bp-long ITS2 and 2696-bp-long partial 28S rDNA. Submission of the newly obtained sequences of 18S or 28S rDNA to BLAST resulted in the identification of genetically related species in the same families, Trichostrongylidae and Chabertiidae. However, the resolution of these analyses was extremely low due to the paucity of deposited nucleotide sequences and extremely high identities of different species within the same family. For example, in the case of *O. muntiacum* (Chabertiidae: Oesophagostominae Railliet, 1916), *Cyclodontostomum purvisi* (Chabertiidae: Chabertiinae Popova, 1952) was identified as having the closest nucleotide sequences (DDBJ/EMBL/GenBank accession nos AJ920340 and AM039732). These two species showed 99.94% identity (1733/1734; 18S rDNA) with two insertion/deletion (indel) sites and 99.59% identity (2685/2696; 28S rDNA). The phylogenetic analyses of genera and/or species of Trichostrongylidae and Chabertiidae nematodes based on the ITS region of rDNA, however, showed a higher resolution (figs 4 and 5) and supported the morphological classification of the present specimens, *C. ninhae* and *O. muntiacum*, in the subfamilies Cooperiinae and Oesophagostominae, respectively.

As a result of different reactivities to the primer pairs used in the reactions, 375-bp- and 852-bp-long *cox-1* nucleotide sequences were obtained for *C. ninhae* and *O. muntiacum*, respectively. Due to the paucity of deposited *cox-1* nucleotide sequences, which showed high identities to the newly obtained ones, no phylogenetic analyses were conducted.

Discussion

Alien species are organisms introduced intentionally or non-intentionally to non-endemic areas of the planet. Invasive species, successfully adapted to their new environments, are of current

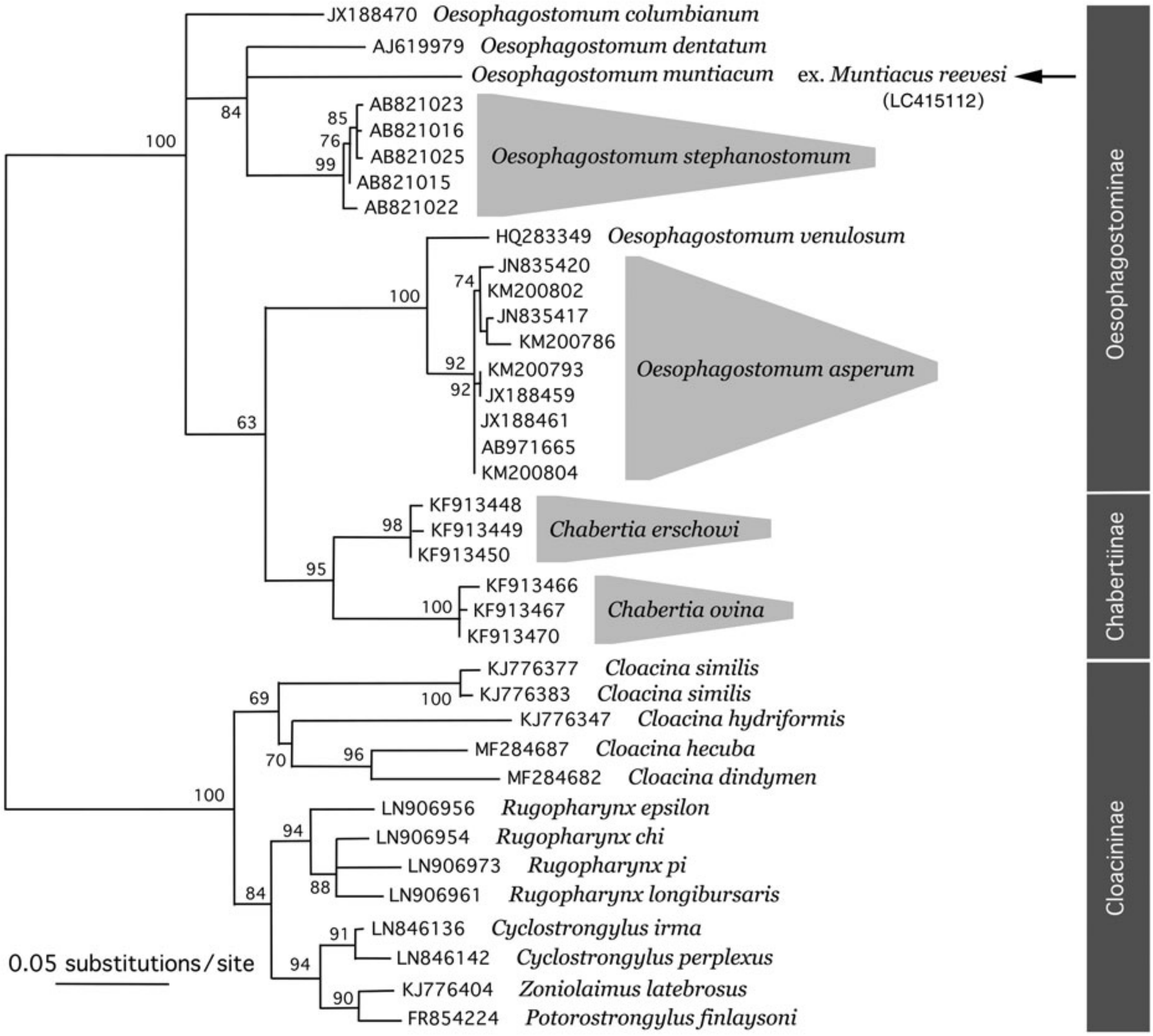

Fig. 5. ML phylogenetic tree based on the ITS regions of rDNA sequence. Representatives of different species of Chabertiidae are shown and they are depicted by their DDBJ/EMBL/GenBank accession numbers. The original analyses were conducted with the following additional sequences: *Oesophagostomum columbianum* (AB908964, AB908965, HQ844232 and JX188471); *Oesophagostomum stephanostomum* (AB821013, AB821014, AB821017–AB821021, AB821024 and AB821026–AB821031); *Oesophagostomum asperum* (JN835418, JN835419, JX188457, JX188458, JX188460, JX188462–JX188467, KM200785, KM200787–KM200792, KM200794–KM200801, KM200803, KM200805 and KM200806); *Chabertia erschowi* (KF913451–KF913456); *Chabertia ovina* (JF680981, KF913468, KF913469 and KF913471); *Cloacina similis* (KJ776380); *Rugopharynx* spp. (LN906955, LN906957–LN906960, LN906962–LN906972 and LN906974); and *Cyclostrongylus* spp. (LN846137–LN846141, LN846143 and LN846144). Arrow indicates the newly obtained rDNA nucleotide sequence of *Oesophagostomum muntiacum* in the present study.

concern due to their impact on the ecological system, including biodiversity and landscapes, economy and health of related organisms (Atkinson, 2001; Courchamp *et al.*, 2003; Robertson *et al.*, 2017). Representatives of invasive alien mammalian species in Japan include the common raccoon (*Procyon lotor*), Taiwan squirrel (*Callosciurus erythraeus*), Siberian chipmunk (*Tamias sibiricus*), hedgehog (*Erinaceus* spp.), brown rat (*Rattus norvegicus*), muskrat (*Ondatra zibethicus*), coypu (*Myocastor coypus*), American mink (*Mustela vison*), Taiwan macaque (*Macaca cyclopis*), etc. (Ohdachi *et al.*, 2010), most of which are invasive in Europe too (Robertson *et al.*, 2017). All-year trapping of invasive alien mammals provides a better opportunity to find and characterize any parasite(s) with an unknown seasonal fluctuation in its prevalence (Sato & Suzuki, 2006; Sato *et al.*, 2006, 2007).

Feral alien Reeves's muntjacs, with a recently expanding population on Izu-Oshima Island, Tokyo, were examined in the present study and three nematode species were detected in their alimentary tracts. In the oesophageal mucosa of the same host individuals, *G. pulchrum* was found in 11 (34.4%) of 32 examined animals. Details about this species are reported in a separate work (Setsuda *et al.*, 2018). Although little is known about the natural parasite fauna of Reeves's muntjacs or, indeed, muntjacs in general in their endemic areas, several valuable taxonomic works have been conducted on trichostrongylid or chabertiid nematodes (Dróżdż, 1967, 1973; Durette-Desset & Chabaud, 1974; Gibbons & Kan, 1987; Jian, 1989). The trichostrongylid and chabertiid species from the feral alien Reeves's muntjacs on Izu-Oshima Island, Tokyo, were identified as *C. ninhae* and *O. muntiacum*, respectively. Some *Oesophagostomum* spp. (Oesophagostominae) and *Chabertia* spp. (Chabertiinae), both subfamilies classified in the family Chabertiidae, were not separated in distinct clades in our phylogenetic tree based on the ITS regions (fig. 5). In accordance, Newton *et al.* (1998) also noticed the same phylogenetic relationships among *Oesophagostomum* spp. and *Chabertia ovina*. This discrepancy between morphological taxonomy and phylogeny has not yet been explained.

Recent mammalogical studies have demonstrated a remarkable diverse speciation of muntjacs due to the recurrent fragmentation of endemic forests in South-East Asia and southern China (Schaller & Vrba, 1996; Giao *et al.*, 1998; Wang & Lan, 2000; Groves & Grubb, 2011; Li *et al.*, 2017). At present, we have no knowledge of the helminth fauna of the different *Muntiacus* spp.; however, they may harbour the trichostrongylid and chabertiid nematodes observed in the present study. Furthermore, it is possible they have additional species such as *Spiculopteragia* spp. (Trichostrongylidae: Ostertagiinae), *Ashworthius tuyenquangi* (Trichostrongylidae: Haemonchinae) and *Trichuris muntjaci* n. comb. (syn. *Trichocephalus muntjaci* Dróżdż, 1970) detected in *M. muntjak* in Vietnam (Dróżdż, 1965, 1970), or related species such as *Hyostrongylus gabonensis* (Trichostrongylidae: Graphidiinae), *Oesophagostomum hyemoschi* and *Toxocara warreni* (Ascarididae: Toxocarinae) identified in *H. aquaticus* in Gabon (Chabaud & Durette-Desset, 1973; Durette-Desset & Chabaud, 1974). Moreover, *Ogmocotyle pygargi* has also been reported from *M. muntjak* in Vietnam (Dróżdż, 1973). The rDNA and *cox-1* nucleotide sequences of muntjac helminths determined here for the first time could help to clarify the relationships of parasite specimens with morphological variations and parasite populations from different *Muntiacus* spp. or host populations with different geographical distributions by allowing us to speculate on the relationships of the newly obtained specimens with archived ones and the possibility of co-evolution of these helminth species with their hosts.

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Conflict of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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