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Eucoleus garfiai (Gállego *et* Mas-Coma, 1975) (Nematoda: Capillariidae) infection in wild boars (*Sus scrofa leucomystax*) from the Amakusa Islands, Japan

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ABSTRACT

We examined lingual tissues of Japanese wild boars (*Sus scrofa leucomystax*) captured in the Amakusa Islands off the coast of Kumamoto Prefecture. One hundred and forty wild boars were caught in 11 different locations in Kamishima (n = 36) and Shimoshima (n = 104) in the Amakusa Islands, Japan between January 2016 and April 2018. Lingual tissues were subjected to histological examinations, where helminths and their eggs were observed in the epithelium of 51 samples (36.4%). No significant differences in prevalence were observed according to maturity, sex or capture location. Lingual tissues positive for helminth infection were randomly selected and intact male and female worms were collected for morphological measurements. Based on the host species, site of infection, and morphological details, we identified the parasite as *Eucoleus garfiai* (Gállego *et* Mas-Coma, 1975) Moravec, 1982 (syn. *Capillaria garfiai*). This is the first report from outside Europe of *E. garfiai* infection in wild boars. Phylogenetic analysis of the parasite using the 18S ribosomal RNA gene sequence confirmed that the parasite grouped with other *Eucoleus* species, providing additional nucleotide sequence for this genus. Since wild boar populations are widely distributed in Japan, continuing surveys on the epidemiology of the parasite and identifying possible intermediate host candidates are crucial for elucidating the transmission route of the parasite.

1. Introduction

In this study, we examined lingual tissues of Japanese wild boars (*Sus scrofa leucomystax*) captured in the Amakusa Islands off the coast of Kumamoto Prefecture, Japan. Wild boars can cause significant damage to agriculture lands and forests. Therefore, the local government and farmers are promoting targeted hunting to reduce the expanding population. By-products from hunting, including game meat, are distributed among consumers through retailers and restaurants, and have become increasingly popular in recent years [1]. Thus, surveying possible zoonotic diseases in the wild boar population is crucial from the public health perspective and for effective population management. The primary reason for inspecting lingual tissues in the present study was to survey for *Sarcocystis* infection, as infection with *S. miescheriana* was reported in the muscles of wild boars, with 50% prevalence [2]. Unexpectedly, during the survey, helminth infections were identified in the lingual tissues. Wild boars in Japan are well known to serve as definitive or paratenic hosts for several helminth species, and this list includes species such as *Paragonimus westermani, Gnathostoma doloresi, Macracanthorhynchus hirudinaceus* and *Gongylonema pulchrum* [3–7]. Here, we report the addition of *Eucoleus garfiai* (Gállego *et* Mas-Coma, 1975) Moravec, 1982 (syn. *Capillaria garfiai*) to this list of helminths found in wild boars in Japan.

2. Materials and methods

2.1. Histological and parasitological examinations

One hundred and forty wild boars were caught in 11 different locations in Kamishima (n = 36) and Shimoshima (n = 104) in the

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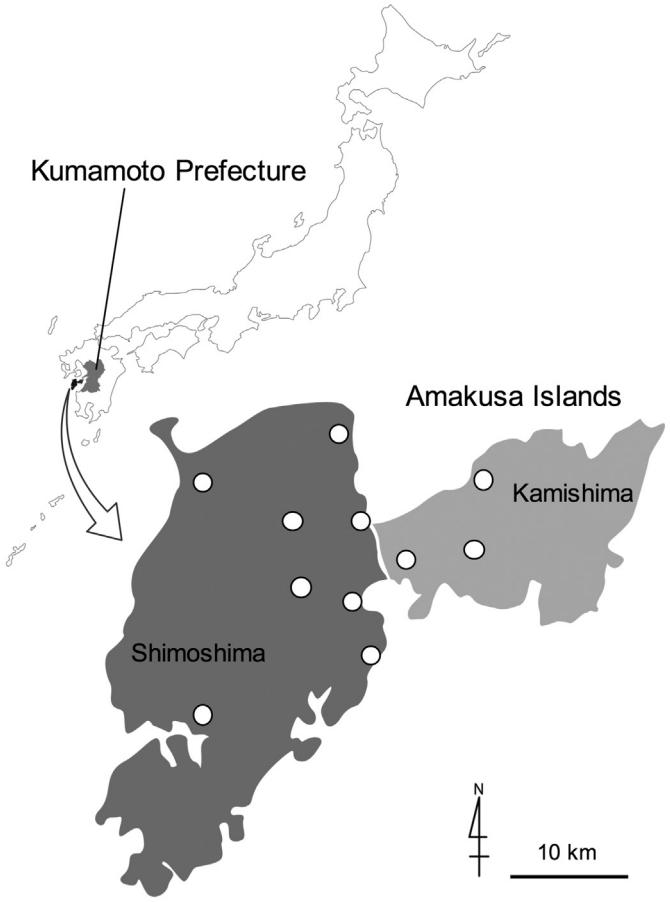

Fig. 1. Map of Amakusa Islands, Japan. One hundred and forty wild boars were caught in 11 different locations, indicated by ○.

Amakusa Islands (Fig. 1) between January 2016 and April 2018. The animals were dissected and the tongues were sent to the Laboratory of Veterinary Pathology, Nihon University, originally to be inspected for *Sarcocystis* infection. The results for *Sarcocystis* infection will be reported separately. Lingual tissues were cut and fixed in 10% neutral buffered formalin and the remaining tissues kept at −30 °C until use. Tissues were embedded in paraffin, cut into 5 μm sections, stained with hematoxylin and eosin, and observed under light microscopy. A single histological section per animal was examined. A chi-squared test was performed to compare the prevalence according to maturity (piglets < 1 year *vs* adults ≥ 1 year), sex (males *vs* females), and the capture location (Kamishima *vs* Shimoshima) of the wild boars. *P* values < .05 were considered significant.

Lingual tissues positive for helminth infection were randomly selected and whole worms (15 males and 15 females, all intact) were collected from the remaining frozen samples by scraping the epithelium of the dorsal surface. The collected worms were washed in saline and preserved in 70% ethanol. Morphological measurements of the worms were made under direct microscopic observation. Measurements for this experiment are described as the range, with mean and standard deviation in the parentheses.

2.2. Phylogenetic analysis

DNA of the collected 3 male and 3 female worms were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacture's protocol. The following PCR primer sets were used for 18S ribosomal RNA (18S rRNA) gene; 1F: 5′-CTGGTTGATCCTGCC AGT-3′ and 536R 5′-GWATTACCGCGGCKGCTG-3′, Nem_18S_F: 5′-CGCGAATRGCTCATAACAACAGC-3′ and Nem_18S_R: 5′-GGGCGGT ATCTGATCGCC-3′, 18S 965: 5′-GGCGATCAGATACCGCCCCTAGTT-3′ and 18S 1573R: 5′-TACAAAGGGCAGGGACGTAAT-3′ [8–10]. PCR was performed in a 50-μL reaction volume, including 1 μL of DNA template, 1× Ex Taq Buffer, 1.25 U of Takara Ex Taq, 0.2 mM dNTP mixture (Takara Bio Inc., Japan), and 0.2 μM primers (Sigma-Aldrich, USA). The reaction conditions included an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 54 °C for the Nem_18S primers and at 53 °C for the 1F/536R and 965/1573R primers, and extension at 72 °C for 60 s, with a final extension at 72 °C for 4 min [9,10]. Amplified products were visualized on a 1.5% agarose gel. Positive PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Germany) according to the manufacturer's protocol, and directly sequenced at a sequencing facility (FASMAC Co. Ltd., Japan) using the same primers as for the PCR.

The obtained sequence was aligned with available sequences of Family Trichuridae and Capillariidae from GenBank using Clustal W implemented in MEGA7 [11]. Phylogenetic relationships were inferred by the maximum likelihoods (ML) method using MEGA7. A Kimura 2-parameter model incorporating a gamma distribution and invariant sites was selected as a substitution model based on the Bayesian information criterion. The phylogenetic tree was obtained after bootstrap analysis with 1000 replications. The sequence obtained in this study was deposited in GenBank under accession number LC484432.

3. Results and discussion

Helminth structures and eggs were observed in 51 (36.4%) of the 140 samples examined histopathologically (Fig. 2). There was no significant difference in prevalence according to maturity (piglets 30/82, 36.6% *vs* adults 21/58, 36.2%), sex (females 22/70, 31.4% *vs* males 29/70, 41.4%), or capture location (Kamishima 14/36, 38.9% *vs* Shimoshima 37/104, 35.6%). Worms and eggs were both confined to the dorsal epithelium of the tongue. Worms were found in prickle cell layers and measured approximately 70 μm in diameter in cross-section. Barrel-shaped eggs were found in a range of keratinized layers and prickle cell layers of the epithelium and measured approximately 50 μm longitudinally. Although no obvious gross pathological changes were observed in the infected tissues, hyperkeratosis of mucosal epithelium and infiltration of lymphocytes, plasma cells, and eosinophils in the mucosa and submucosa were observed histologically (Fig. 2).

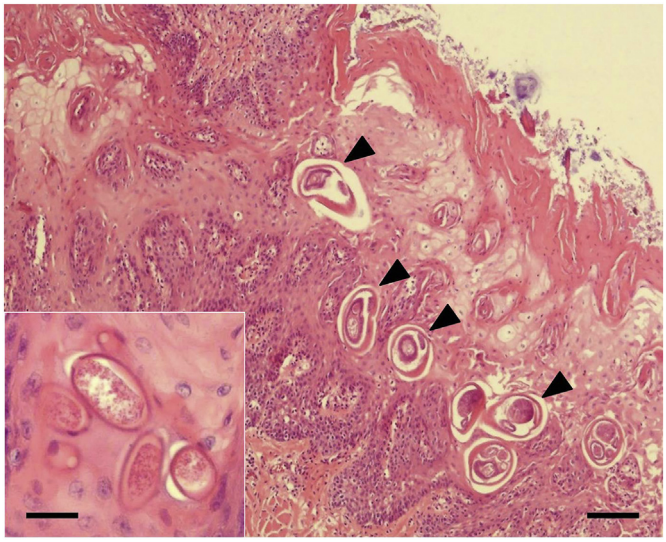

Fig. 2. Histopathology of the dorsal lingual epithelium. Adult worms (arrowhead, bar = 100 μm) and eggs (inset, bar = 30 μm) embedded in the epithelium. A large number of inflammatory cell infiltrates could be observed in the submucosa.

Table 1
Morphological measurements of Capillariidae species collected from the lingual epithelium of *Sus scrofa*. Modified the table by Löwenstein and Kutzer [14] and added data from this study.

	A. papuensis [a)]	*E. garfiai* [b)]	*E. garfiai* [a)]	*E. garfiai* [c)]	*E. garfiai* [c)]	*E. garfiai* [d)]
Reference	[16]	[12]	[13]	[14]	[18]	This study
Host	*S. scrofa papuensis*	*S. scrofa*	*S. scrofa*	*S. scrofa*	*S. scrofa.* (Domestic pig)	*S. scrofa leucomystax*
Locality	Papua New Guinea	Spain	Spain	Austria	Austria	Japan
Location of parasite	Tongue	Tongue	Tongue	Tongue	Tongue	Tongue
Male (mm)	$n = 25$	$n = 6$ (fragments)	$n = 10$	$n = 30$	$n = 2$	$n = 15$
Body length	10.5–14.5 (13.2)	7.0–8.0	11.945–13.149 (12.433)	8.11–12.39	10.95–11.00	6.84–10.73 (9.50 ± 1.11)
Maximum body width	0.051–0.061 (0.057)	0.061	0.083–0.095 (0.091)	0.060–0.095	0.08–0.10	0.062–0.087 (0.070 ± 0.008)
Esophagus length	4.80–5.92 (5.49)	3.45	3.797–4.491 (4.044)	2.55–3.85	2.70–3.56	2.79–3.60 (3.19 ± 0.21)
- Length of muscular esophagus	–	0.24	0.274–0.370 (0.314)	0.40–0.60	0.50–0.70	0.26–0.63 (0.40 ± 0.12)
- Length of stichosome	–	3.21	3.478–4.121 (3.730)	2.15–3.25	2.20–2.86	2.51–3.28 (2.76 ± 0.21)
- No. of Stichocyte	–	37	–	28–30	29–30	23–30 (26.13 ± 2.42)
Posterior body length	–	3.530–3.970	7.870–8.797 (8.389)	5.56–8.54	7.44–8.25	3.85–7.17 (6.31 ± 1.01)
Esophagus: Posterior body ratio	–	1:1	1:1.84–2.25 (2.07)	1:1.7–2.2	1:2.09–3.05	1:1.31–2.35 (1.98 ± 0.30)
Spicule	Present	Not present	Present	Not present	Not present	Presence not confirmed
- Length	0.602–0.865 (0.680)	–	–	–	–	–
Spicule sheath	Smooth	With spines	With spines	With spines	With spines	With spines
- Length	–	0.89–0.98	1.262–1.459 (1.368)	0.9–1.25	0.95	0.81–1.16 (0.99 ± 0.11)
Lateral wing	Present	–	–	–	Not present	Not present
- Size	0.187–0.33 × 0.003–0.004	–	–	–	–	–
Caudal wing	Present	Not present	–	Present, very narrow	Very small	Presence not confirmed
Caudal end	Two rib-shaped extensions	Two lateral lobes	–	Two ball-like structures	Two ball-like structures	Two ball-like structures
Papillae	–	one, ventral	–	Numerous, irregulary distributed, different sizes	–	Presence not confirmed
Anus	–	–	–	Terminal	Terminal	Terminal
Female (mm)	$n = 31$	$n = 6$ (fragments)	$n = 10$	$n = 20$	$n = 6$	$n = 15$
Body length	13.1–23.5 (18.4)	9–10	16.158–17.223	11.03–15.16	14.70–17.35	11.02–14.80 (12.76 ± 1.01)
Maximum body width	0.058–0.086 (0.070)	0.081	0.104–0.122 (0.111)	0.055–0.1	0.095–0.125	0.074–0.107 (0.093 ± 0.011)
Esophagus length	5.04–6.72 (5.89)	4.05	3.611–4.352 (3.940)	2.75–4.20	3.38–4.18	2.94–3.42 (3.16 ± 0.16)
- Length of muscular esophagus	–	0.32	0.334–0.471 (0.394)	0.46–0.63	0.77–1.10	0.36–0.81 (0.48 ± 0.11)
- Length of stichosome	–	3.73	3.140–4.018 (3.546)	2.29–3.57	2.40–3.10	2.34–2.89 (2.65 ± 0.17)
- No. of Stichocyte	–	41	–	30–32	27–31	21–28 (24.20 ± 2.24)
Posterior body length	–	5.18	12.454–14.214 (13.283)	8.25–10.96	11.05–13.49	8.01–11.50 (9.58 ± 0.96)
Esophagus: Posterior body ratio	–	1:1.279	1:2.98–3.80 (3.38)	1:2.4–3.1	1:2.64–3.47	1:2.63–3.55 (3.03 ± 0.29)
Esophagus - vulva distance	0.072–0.210 (0.143)	0.011–0.035	0.025–0.079 (0.50)	0.04–0.08	0.05–0.09	0.034–0.091 (0.064 ± 0.019)
Papillae	16 large papillae grouped in two positions near the opening of the vuvla	–	–	Numerous, irregulary distributed, different sizes	–	Presence not confirmed
Anus	Subterminal	Subterminal	–	Subterminal	Subterminal	Subterminal
Egg (μm)					$n = 40$	$n = 73$
Size	51–65 (61) × 20–27 (24)	54–64.8 × 27–28.8	61.2–68.4 (64) × 27–30.6 (28.6)	59.3–71.44 × 25.8–38	59.4–77.9 × 26.5–30.7	47.4–56.2 (51.8 ± 2.4) × 20.9–28.8 (24.8 ± 2.3)
Surface	Rough, vertical stripes	–	Irregular striation, longitudinally oriented	–	Uneven, without clear structure	–

Measurements are presented as, a) range with mean in the parentheses, b) mean, c) range and d) range with mean and standard deviation in the parentheses.
–: data not available.

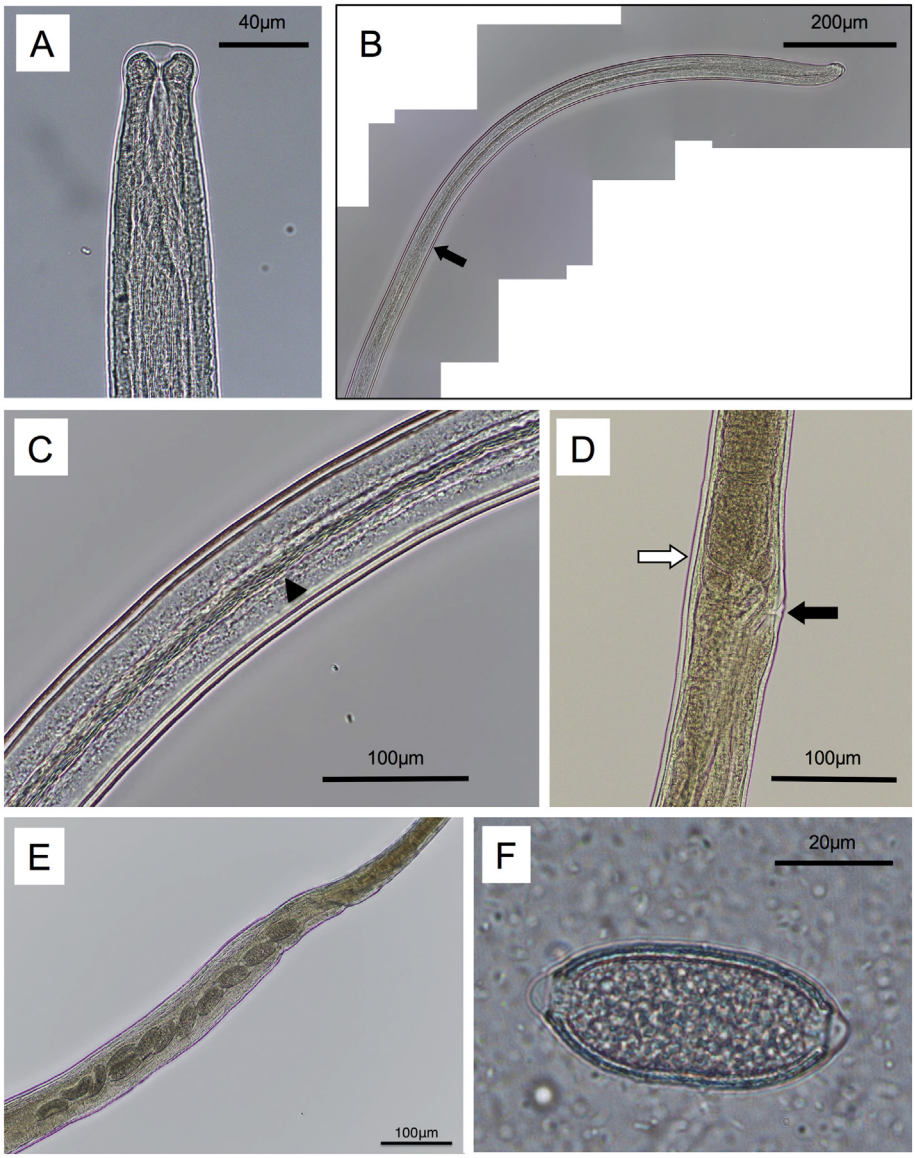

Fig. 3. A: Ventral view of the caudal end of the male worm. B and C: Posterior body of the male worm (lateral view). The arrow in B indicates the start of the spicule sheath with dense spines. Details of the spicule sheath (arrowhead) is shown in C. D: Vulvar region of the female worm. The arrow indicates the distance from the esophageal end (white arrow) to the vulva (black arrow). E: An egg-containing uterus of the female worm. F: A released egg.

Morphological measurements of the parasite are described in Table 1, along with the data from previous studies [12–18]. Male: The caudal end had two lateral lobes, connected by a reduced membrane (Fig. 3A). Spicule sheaths with dense spines, which were 0.81–1.16 mm (0.99 ± 0.11 mm) in length, were observed in all specimens (Fig. 3B and C). We could not confirm the presence of the spicule. Female: Vulvae were located 0.034–0.090 mm (0.064 ± 0.019 mm) from the end of esophagus and openings were not elevated (Fig. 3D). Eggs were arranged in a single row in the uterus, unembryonated, barrel-shaped, with polar plugs at both ends (Fig. 3E and F), and measured 47.4–56.2 × 20.9–28.8 μm (51.8 ± 2.4 × 24.8 ± 2.3 μm). Caudal ends were rounded with a sub-terminal anus.

These morphological details, in addition to the host species (wild boar) and site of infection (lingual epithelium), were all in agreement with the description of *E. garfiai* Gállego *et* Mas-Coma, 1975 [12–15]. *E. garfiai* can be clearly distinguished from other Capillariidae species reported from *S. scrofa*, including *Aonchotheca papuensis* and *Aonchotheca suis*, by the morphological characteristics proposed by Moravec [15–17]. Notably, *A. papuensis* infection has been reported from the lingual epithelium of *Sus scrofa papuensis*, confirming that two different species from different genera, *E. garfiai* and *A. papuensis*, infect the same tissue of *S. scrofa* [16]. Since the first report from Gállego and Mas-Coma in Spain [12], the parasite has also been reported in Austria and France [14,18–20], but not in any other part of the world. This is the first report of *E. garfiai* infection in wild boars outside Europe.

The parasite is suggested to be widely prevalent in the wild boar population of the Amakusa Islands irrespective of maturity, sex or habitat. However, the infection rate was lower (36.4%) than that reported in previous studies, 82% [13] and 69% [14]. It is possible that actual infection rate in the Amakusa Islands is higher than what we have observed, since the result here is based on histological examination of a single section per animal. Indeed, infiltration of the inflammatory cells in the lingual submucosa was observed for some of the sections that were negative for *E. garfiai*, which may indicate the presence of the parasite. Nevertheless, histological observations, both here and in previous studies [12,14,19], suggest that the pathogenicity of the parasite is negligible.

Although the presence of a sclerotized spicule is noted as a morphological description of *Eucoleus* species, studies describing *E. garfiai* [12,14,18] did not report such a morphological feature, except for the

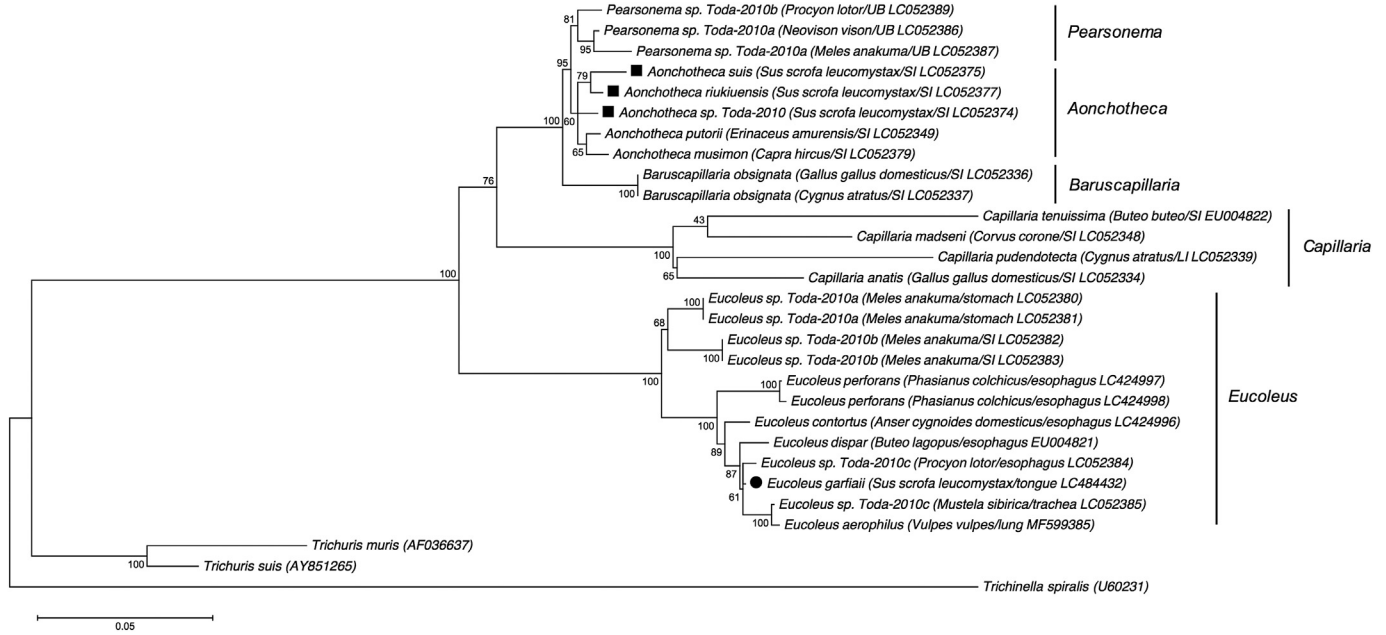

Fig. 4. Phylogenetic relationships among Capillariidae inferred from partial 18S rRNA gene sequence using the maximum likelihood method, with bootstrap proportion values. The Kimura 2-parameter model incorporating a gamma distribution and invariant sites was selected as a substitution model based on the Bayesian information criterion. The consensus tree was obtained after bootstrap analysis with 1000 replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1453 positions in the final dataset. *Eucoleus garfiai* in the present study is indicated by ●, whereas other Capillariidae species from *Sus scrofa* are indicated by ■. UB, urinary bladder; SI, small intestine; LI, large intestine.

study by Gállelo et al. [13]. In this latter work, the authors were able to distinguish the presence of a fine spicule, partially protruding from the sheath, under phase contrast microscopy. Unfortunately, we could not confirm the presence of a spicule here. It is reported that spicule for *Eucoleus* species is often difficult to distinguish [15,21], reflecting the difficulty in differentiating family Capillariidae by morphology, which is currently divided into 27 genera containing > 300 species [21].

Although there is insufficient information on nucleotide sequences for Capillariidae species, molecular phylogenetic analysis based on the 18S rRNA and mitochondrial cytochrome *c* oxidase subunit genes has supported the morphological classification proposed by Moravec [15] to a certain extent [17,22]. Therefore, we conducted a phylogenetic analysis of *E. garfiai* using the 18S rRNA gene sequence to characterize this species genetically for the first time. A phylogenetic ML tree based on the 18S rRNA gene sequences of representative Capillariidae species is shown in Fig. 4. *E. garfiai* grouped with other *Eucoleus* species and was separated from the genus *Aonchotheca*, which underpins the morphological classification [15,21].

The genus *Eucoleus* has been reported in carnivores and birds, and members of this genus are known to parasitize either the respiratory systems or the epithelium of the upper digestive tract [21]. *E. aerophilus* and *E. boehmi* are relatively known in Europe, where they are reported to cause respiratory capillariosis in domestic dogs and cats by infecting the epithelium of upper respiratory organs [23]. *Eucoleus* species that parasitize in the epithelium of the oral cavity, esophagus, and stomach have been reported in mammals, including the rat (*Rattus norvegicus*), raccoon (*Procyon lotor*), and the Japanese badger (*Meles anakuma*) [17,24,25]. *E. procyonis* was originally reported in the esophagus of raccoons in America [15,26]. Later, the parasite was also found in the lingual epithelium, demonstrating histopathology similar to that observed for *E. garfiai* infection [27]. Similarly, in birds of prey, *E. dispar* is found in the epithelium of the esophagus, but is also known to parasitize the tongue [28,29]. These descriptions suggest that the tongue, along with the esophagus, may be a common infection site for some of the parasites in the genus *Eucoleus*. To date, the infection site for *E. garfiai* is reported to be strictly limited to the tongue [13]. However, we did not examine other areas of the upper digestive tract in the present study and further screening may be necessary to confirm this.

The parasite eggs are thought to be released into the oral cavity when the epithelial cells are being shed and then excreted in the feces, although previous studies have shown both positive and negative results for fecal examinations [14,18,19]. Earthworms, including *Lumbricus terrestris*, *Allolobophora caliginosa*, and *A. rosea*, are reported to be involved in maintaining the life cycle for *E. garfiai* as intermediate hosts, where they are assumed to become infected through contaminated soil [18]. Feeding earthworms collected from the wild boar habitats to domestic pigs has been shown to establish the infection, while providing embryonated egg or contaminated soils failed to do so [18]. However, natural infection in domestic pigs is unlikely to occur in Japan where indoor farming systems predominate, with little or no possibility of contact between the infected animals and the intermediate hosts.

The risk of zoonotic transmission of *E. garfiai* to humans is also considered to be low because of their presumed life cycle and the fact that the parasite has not been reported in mammals other than *S. scrofa*. Nevertheless, from a public health perspective, it is important to be aware of the existence of the parasite and to continue surveys on wild boar populations including other areas in Japan to understand the epidemiology of *E. garfiai*. In addition, it is not yet clear how the parasite life cycle is maintained in the population of the Amakusa Islands. Therefore, conducting fecal examinations and unraveling possible intermediate host candidates in the Islands are crucial in elucidating the transmission route of *E. garfiai*.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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