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FIRST MORPHOLOGICAL AND MOLECULAR DESCRIPTION OF *ANATRICHOSOMA* SP. (NEMATODA: TRICHOSOMOIDIDAE) IN THE NASAL CAVITIES OF A SHEEP (*OVIS ARIES*) FROM SARDINIA (ITALY)

Alessia L. Gazzonis[1,2], Giuseppe D'Amico Ricci[3], Maria Teresa Manfredi[1,2], and Giovanni Garippa[4]

[1] Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, via dell'Università 6, 26900 Lodi, Italy.
[2] Research Laboratory of Animal Parasitic Diseases and Zoonoses (ParVetLab), Università degli Studi di Milano, via dell'Università 6, 26900 Lodi, Italy.
[3] U.O. Oculistica, Turin Eye Hospital, ASL Città di Torino, Turin, Italy.
[4] Department of Veterinary Medicine, University of Sassari, Via Vienna, 2, 07100 Sassari, Italy.
Correspondence should be sent to Maria Teresa Manfredi (https://orcid.org/0000-0002-9623-9475) at: *mariateresa.manfredi@unimi.it*

KEY WORDS

Anatrichosoma
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Nasal cavities
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Italy
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Mitochondrial cytochrome *c* oxidase subunit I (*COI* mtDNA)

ABSTRACT

Cases of parasitic infection by *Anatrichosoma* spp. have occasionally been reported worldwide in a narrow range of host species, including humans. The species in which *Anatrichosoma* has been most commonly reported are nonhuman primates; among domestic animals, infections have only been reported in dogs and cats. Many aspects of the biological cycle and epidemiology of the parasite are still unknown. In the present study, we report the case of *Anatrichosoma* sp. infection in the nasal cavities of a sheep (*Ovis aries*) raised in Sardinia (Italy). After sagittal cutting of the sheep's head, 12 adult nematodes were recovered and collected: 10 specimens were subjected to morphological identification; 2 other specimens were processed for deoxyribonucleic acid (DNA) extraction and polymerase chain reaction targeting the small subunit (*18s*) ribosomal ribonucleic acid and the mitochondrial cytochrome *c* oxidase subunit I (*COI* mtDNA). Morphological identification classified all specimens as adult females belonging to the genus *Anatrichosoma*. Phylogenetic analysis placed the obtained sequences for both genes in the cluster of *Anatrichosoma* spp., with a higher interspecific variation observed at the *COI* mtDNA gene. The present unusual case of *Anatrichosoma* sp. infection in a sheep adds a new suitable host for this uncommon parasite, providing novel genetic data. This finding suggests that this rare parasite should be considered in human and animal mucosal parasitic infections and that molecular diagnosis should be performed in equivocal or doubtful cases.

Anatrichosoma is a rarely detected genus of trichuroid nematodes belonging to the Subfamily Anatrichosomatinae (Order: Trichocephalida; Superfamily: Trichinelloidea; Family: Trichosoimoididae) (Hodda, 2022). Five species have been described, including *Anatrichosoma buccalis* (Pence and Little, 1972; Kinsella and Winegarner, 1975), *Anatrichosoma haycocki* (Spratt, 1982), *Anatrichosoma ocularis* (File, 1974) *Anatrichosoma cynamolgi* (Smith and Chitwood, 1954) (synonyms *Anatrichosoma rhina* Conrad and Wong, 1973, *Anatrichosoma nacepobi* Conrad and Wong, 1973, Long et al., 1976), and *Anatrichosoma gerbillis* (Bernard, 1964).

Anatrichosoma spp. infect a wide range of nonhuman primates (NHPs) of the Old and New World (chimpanzees, cynomolgus and rhesus monkeys, lesser spot-nosed monkeys, pig-tailed macaques, tongue macaques, proboscis monkeys, sooty mangabey, vervet monkeys, white-handed gibbons) (Breznock and Pulley, 1975; Ulrich et al., 1981; Petrzelkova et al., 2006, Petrášová et al., 2010; Kouassi et al., 2015; Klaus et al., 2018; Choong et al., 2019; Thilakarathne et al., 2021), a few rodents (gerbils), common tree shrews (Bernard, 1964; File, 1974), and marsupials (Swainson's and brown marsupial mice, common opossums) (Pence and Little, 1972; Kinsella and Winegarner, 1975; Spratt, 1982). In addition, unidentified *Anatrichosoma* sp. have been described in cutaneous lesions resembling those of larva migrans (Morishita and Tani, 1960; Hoa et al., 1963; Marwi et al., 1990), in the oral mucosa (Eberhard et al., 2010, 2014) and in human breast nodules (Pampiglione et al., 2005). Finally, infections considered accidental affect dogs and cats (Lange et al., 1980; Hendrix et al., 1987; Jitsamai et al., 2021).

There seems to be site specificity toward the mucosa of vertebrates in parasites of this genus. Infections can cause lesions that have been described in several mammalian hosts. For example, lesions caused by *Anatrichosoma* spp. in the mucosa and submucosa of nasal cavities have been described in NHPs, particularly *A. cynamolgi* in the rhesus monkey (*Macaca mulatta*) (Conrad

and Wong, 1973), *Anatrichosoma* sp. in cynomolgus macaque (*Macaca fascicularis*) (Takenaka et al., 1989), and *A. buccalis* in the common opossum (*Didelphis marsupialis*) (Pence and Little, 1972).

Anatrichosoma spp. have also been reported in various anatomical structures, e.g., unidentified *Anatrichosoma* in the ears, lips, nares, and eyelids of white-handed gibbons (*Hylobates lar*) (Breznock and Pulley, 1975). *Anatrichosoma haycocki* has been found in the paracloacal gland of the Swainson's marsupial mouse (*Antechinus swainsonii*) and brown marsupial mouse (*Antechinus stuartii*) (Spratt, 1982). In addition, *A. buccalis,* which is known to cause pododermatitis, has been described in opossums (*D. marsupialis* and *Didelphis virginiana*) (Kinsella and Winegarner, 1975).

In domestic animals, *Anatrichosoma* sp. has been found in the ear canal of a dog with purulent otitis externa (Hendrix et al., 1990), in the nodule on the dorsal midline in the lumbar region of a dog (Hendrix et al., 1987), and in cats with ulcerative pododermatitis (Lange et al., 1980; Ramiro-Ibanez et al., 2002; Noden et al., 2013; Jitsamai et al., 2021).

In the cases described in the literature, the diagnosis of *Anatrichosoma* spp. infection is often based on the identification of adult parasites in the pathological lesions, or on the examination of histological sections of surgical biopsy specimens (e.g., Pampiglione et al., 2005; Eberhard et al., 2014) and, to a lesser extent, on the detection of eggs in fecal samples (Klaus et al., 2018; Choong et al., 2019; Thilakarathne et al., 2021). Only in a few cases was molecular confirmation performed in addition to morphological identification (Zhu et al., 2000; Eberhard et al., 2014; Jitsamai et al., 2021); consequently, genetic data are scarce, with only 4 nucleotide sequences of *Anatrichosoma* spp. available in GenBank (KU215886, OK044796, KF581196, and AJ288160; accessed 7 July 2023).

The present study describes an unusual case of *Anatrichosoma* sp. infection in the nasal cavities of a sheep (*Ovis aries*) bred in Sardinia (Italy), with the morphological description of adult female specimens and new genetic data.

MATERIALS AND METHODS

The infected animal described in the present study was an adult female dairy sheep of the Sarda breed, born and raised in a farm located in northwestern Sardinia (Italy) and destined for slaughter in a local certified abattoir. However, the infection was also found in other subjects, with an estimated prevalence of around 100%. The nematodes described hereafter were recovered in the nasal cavities of the sheep during a sampling campaign aimed at collecting *Oestrus ovis* larvae (D'Amico Ricci et al., 2021). Specifically, 12 specimens were collected after cutting the sheep's head in half along the sagittal axis. The nematodes were preserved in 70% ethanol, and 10 of them, after clarification with lactophenol, were mounted on temporary slides and examined with a light microscope (Axioskop 2, Carl Zeiss, Milan, Italy) equipped with a digital camera. All measurements are given in micrometers or millimeters; the mean value is given, followed by the standard deviation and range values. Specimens were deposited in the Parasitological Collection of the Manter Laboratory of Parasitology (https://hwml.unl.edu/) (collection number: HWML-119226).

Morphological identification of nematodes to genus level was carried out according to Pence and Little (1972) and Spratt (1982). Data from Chitwood and Smith (1958), Bernard (1964), and File (1974) were also used.

Molecular analysis

Two specimens were used for the molecular analysis. Genomic deoxyribonucleic acid (DNA) was extracted using NucleoSpin® tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. A sequence of the small subunit ribosomal ribonucleic acid (*18s* rRNA) gene and of the mitochondrial cytochrome *c* oxidase subunit I (*COI* mtDNA) gene were amplified by polymerase chain reactions (PCRs) according to the described protocols (Powers et al., 2009; Guardone et al., 2013). Each sample was analyzed in duplicate; any positive control of *Anatrichosoma* sp. DNA was not used for PCR amplifications; distilled water was used as the negative control.

Obtained amplicons were purified with a commercial kit (NucleoSpin® gel and PCR clean-up kit, Macherey-Nagel GmbH and Co. KG) and sent for bidirectional sequencing to a commercial service (Microsynth, Balgach, Switzerland). The quality of the sequences was checked using the electropherograms, and consensus sequences were manually assembled. Sequences were compared with those deposited using BLASTn (https://blast.ncbi.nlm.nih.gov/, accessed 6 October 2022) (Camacho et al., 2009), and then aligned with sequences of partial *18s* rRNA and *COI* mtDNA of 7 and 6 species, respectively, of Trichuroidea available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using MEGA version X (Kumar et al., 2018).

Phylogenetic analysis

Phylogenetic trees from the sequences of partial *18s* rRNA and *COI* mtDNA were constructed with the maximum likelihood method using the Kimura 2-parameter for the substitution model, using MEGA X software (Kumar et al., 2018). To evaluate the branch support, the bootstrap method was used with 1,000 replicates. Sequences of partial *18s* rRNA and *COI* mtDNA of 7 and 6 species, respectively, of Trichuroidea were used; *Haemonchus* sp. (DQ503465) for *18s* rRNA and *Teladorsagia circumcincta* (MW051267) for *COI* mtDNA were set as outgroups. For both genes, to estimate the evolutionary divergence between the sequences obtained in the present study and the other available sequences of *Anatrichosoma* spp., the number of nucleotide base substitutions per site was calculated using the Kimura 2-parameter model using MEGA X software (Kumar et al., 2018).

RESULTS

All nematodes collected were identified as adult females of *Anatrichosoma* sp. The following measurements are from 10 specimens (all measurements are expressed in micrometers unless otherwise stated). The nematode has a body that increases in diameter from the anterior to posterior; the anterior end is very thin (mean width at nerve ring = 27.66 ± 1.64, 25.5–29.7), thinner than the posterior (mean maximum width = 152.08 ± 1.93, 150.04–154.4). The total length is 8.24 ± 1.58 mm (6.7–10.3 mm). Width near the vulva is 99.08 ± 2.77 (94.89–101.89). The anterior end is 5.86 ± 1.68 mm (3.6–7.9 mm) long (anterior extremity to the esophagointestinal junction) and 63.57 ± 1.42 (61.57–64.99) wide at last stichocyte. Oral opening lacks lips, small buccal capsule with no evidence of a dorsal stylet. Two lateral amphids are present at the anterior end.

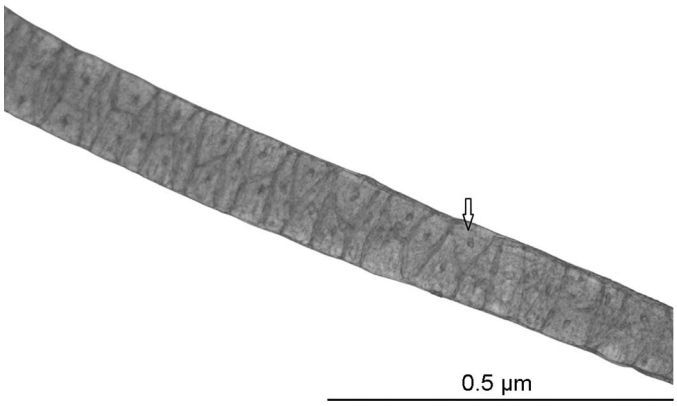

Figure 1. *Anatrichosoma* sp. stichocytes, mid-stichosome (the arrow points to a stichocyte).

Esophagus narrow anteriorly, broadening posteriorly, and narrowing again as it enters the stichosome; mean length of the muscular portion of the esophagus is 182.35 ± 1.64. The stichosome consists of 95–122 stichocytes and may have double stichocytes (Fig. 1). The majority is wider than long, regularly oriented, and has a large nucleus. The vulva, which is nonprotrusible with a prominent appendage, is located 5.23 ± 1.79 mm from the cephalic end; the minimum and maximum of this distance are equal to 3.36 and 6.38 mm, respectively (Fig. 2). Eggs have a mean size of 66.57 ± 2.02 × 46.27 ± 1.87 (64.3–69.6 × 43.8–48.1); dark brown, barrel shaped with polar plugs, thick shelled and contain a first-stage larva in the distal uterus (Fig. 3). Anal opening terminal. A comparison of the measurements of adult females described in the present study with those reported for recognized *Anatrichosoma* species is presented in Table I.

Molecular analyses

For both specimens, molecular analysis produced amplicons of both *18s* rRNA and *COI* mtDNA genes that were successfully sequenced. Sequences from the 2 specimens were identical (100% identity) for both genes. The alignment was constituted by 593 and 399 base pairs when using *18s* rRNA and *COI* mtDNA, respectively. A representative sequence for both genes was deposited in GenBank under the accession codes OQ842899 and OQ834908 and then used for the subsequent phylogenetic analysis.

A BLAST comparison found 98.47% identity with *Anatrichosoma* sp. from the olive glass mouse (*Abrothrix olivacea*) (KU215886), confirming the morphological identification of *Anatrichosoma* sp.

Phylogenetic analysis performed on the sequences of both *18s* rRNA and *COI* mtDNA showed the presence of 4 clusters: *Capillaria* spp., *Trichuris* spp., *Trichinella* spp., and *Anatrichosoma* spp.

The sequence of *18s* rRNA gene of *Anatrichosoma* obtained in the present study was placed in the cluster of *Anatrichosoma* spp., together with *Anatrichosoma* sp. from the olive glass mouse (KU215886) and *Anatrichosoma* sp. from a domestic cat (*Felis catus*) (OK044796) (Fig. 4). Similarly, for *COI* mtDNA the cluster including the sequence obtained in the present study contained *Anatrichosoma* sp. from the olive glass mouse (KF581196) and *A. haycocki* from the Australian bush rat (*Rattus fuscipes*) (AJ288160) (Zhu et al., 2000) (Fig. 5).

Among the available sequences of *Anatrichosoma* spp., the minimum nucleotide difference was observed at *18s* rRNA gene (0.5% and 3.6% between our sequence and the KU215886 and OK044796, respectively), whereas a higher interspecific variation was observed at the *COI* mtDNA gene (29.3% and 32.8% between our sequence and KF581196 and AJ288160, respectively).

DISCUSSION

The present study reports the first case of infection by *Anatrichosoma* sp. in the nasal cavities of a sheep reared in Sardinia (Italy), with morphological identification supported by genetic data.

Parasitic infections in the nasal cavities of mammals are usually caused by arthropods or pentastomids (Taylor et al., 2016). In this localization, nematodes are less frequently detected, with few

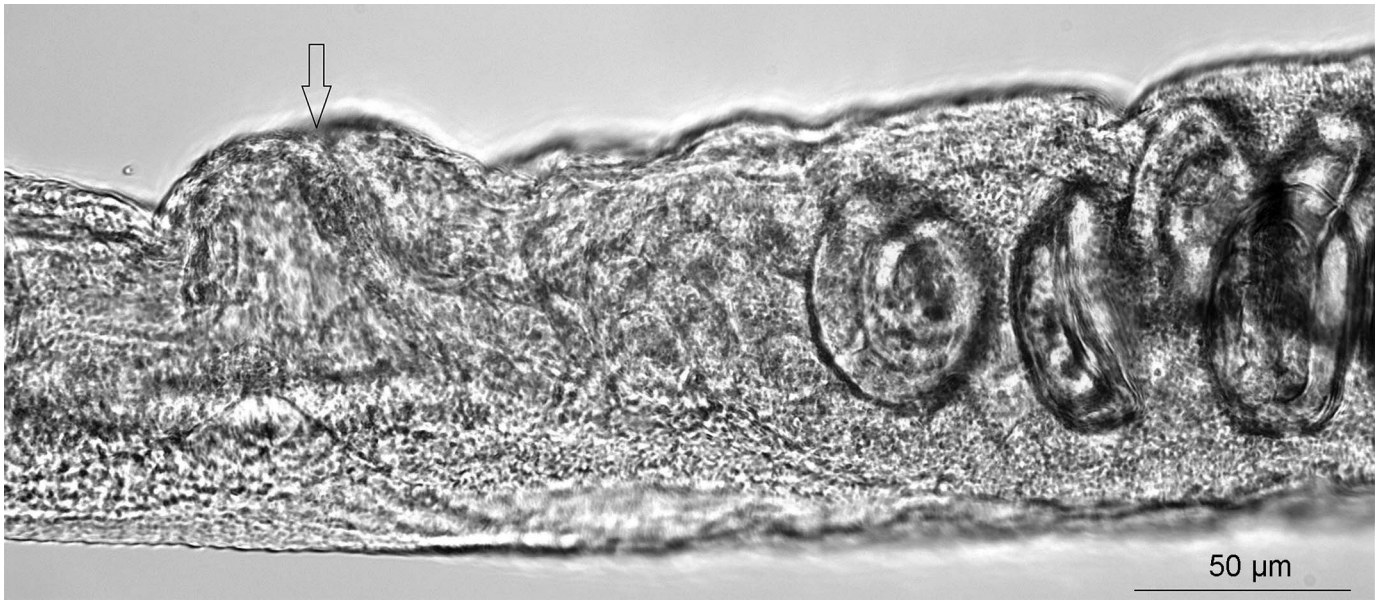

Figure 2. *Anatrichosoma* sp., vulvar region, lateral view (the arrow points to a vulvar opening).

Figure 3. *Anatrichosoma* sp. larvated eggs in the uterus (the arrows point to a larva in the egg).

representatives able to parasitize nasal cavities, e.g., *A. cynamolgi, Trichosomoides nasalis*, *Eucoleus boehmi* (Trichuroidea), and *Nasistrongylus antechini* (Trichostrongylidae) (Durette-Desset and Beveridge, 1981). Because of their peculiar localization in the nasal mucosa, these parasites may cause pathological lesions with possible negative sequelae on the olfaction and thus on the behavior of the host (Veronesi et al., 2013).

Anatrichosoma spp. have previously been reported in the nasal cavities of NHPs (*A. cynamolgi* and *Anatrichosoma* sp.) (Conrad and Wong, 1973; Takenaka et al., 1989). With the detection of *Anatrichosoma* sp. in the nasal cavities of a sheep, the present study adds a new suitable host for this nematode and a new parasite to be included in the differential diagnosis for parasitic infection of the nasal cavities of sheep.

In the current investigation, we could not identify the recovered nematodes to the species level because only adult females were detected in the sheep examined. Although the life cycle of *Anatrichosoma* spp. is poorly understood, females are found in the superficial layers of the mucosa, whereas males are located in the submucosal layer (Little and Orihel, 1972). The morphometric analysis performed on the collected specimens agrees with previous descriptions of *Anatrichosoma*. Moreover, a few differences from the other members of the genus were observed. Female specimens of *Anatrichosoma* sp. found in sheep are shorter and are easily distinguished by the absence of a pronounced cephalic cuticular inflation, the different number of stichocytes, and the smaller ratio of the anterior body region to the total body. Furthermore, the *Anatrichosoma* recovered from the nasal cavities of *Ovis aries* occurred outside the distribution range of other *Anatrichosoma* species, which are more commonly reported from Africa, Asia, and Australia (see Table I). *Anatrichosoma* sp. described here most closely resembles *A. ocularis*; it can be distinguished from the latter species by its shorter total length, greater esophageal length and distance from the vulva to the anterior end, and greater number of stichocytes.

Table I. Comparison of adult females belonging to *Anatrichosoma* species by main measurements (all in millimeters unless otherwise indicated). Minimum and maximum values are usually given and the mean (in parentheses) is given only when reported by the author. Data include habitat, host, and locality where nematodes were collected.

Species	*Anatrichosoma cynamolgi*	*Anatrichosoma gerbillis*	*Anatrichosoma buccalis*	*Anatrichosoma ocularis*	*Anatrichosoma haycocki*	*Anatrichosoma* sp.
Reference	Chitwood and Smith, 1958	Bernard, 1964	Pence and Little, 1972	File, 1974	Spratt, 1982	Present work
Total length	17.8–19.6	27.0–35.1	17.3–34.0 (25.1)	8.0–11.4 (9.16)	6.4–19.9 (11.7)	6.7–10.3 (8.2)
Length of anterior end	5.6	3.3–4.0	2.9–4.9 (3.7)	—*	—	3.6–7.9 (5.8)
Ratio of anterior end/total length	1:3–1:6	1:7.1–1:10	1:5–1:8.3	—	—	1:1.4
Number of stichocytes	126–132	—	71–93	67–74	71–85	95–122
Egg length	70–75 µm	68–77 µm	53–70 (61) µm	70–75 (72.5) µm	69–79 (75) µm	64.3–69.6 (66.5) µm
Egg width	45–57 µm	31–50 µm	33–40 (37) µm	39–44 (72.5) µm	44–46 (45) µm	43.8–48.1 (46.2) µm
Habitat	Mucosa of roof and sides of nasal vestibule, females in stratified epithelium, males in lamina propria	Stomach mucosa	Mucosa of hard palate, gums, and tongue	Squamous epithelium of sclera, cornea, and palpebral conjunctivae	Mature males in epithelial lining of paracloacal glands or in connective tissue around glands, mature females free in paracloacal glands or encapsulated in lumen of cloaca. Immature males and females free in intestine	Mucosa of nasal cavities
Host	*Macaca cynamolgi* and *Macaca mulatta*	*Gerbillus pyramidum*	*Didelphis virginiana*	*Tupaia glis*	*Antechinus swainsonii* and *Antechinus stuartii*	*Ovis aries*
Locality	Asia, Philippines	Africa, Tunisia	America, Louisiana, Costa Rica, and Colombia	Asia, Thailand	Australia, New South Wales	Europe, Italy

* - (hyphen) = information not available.

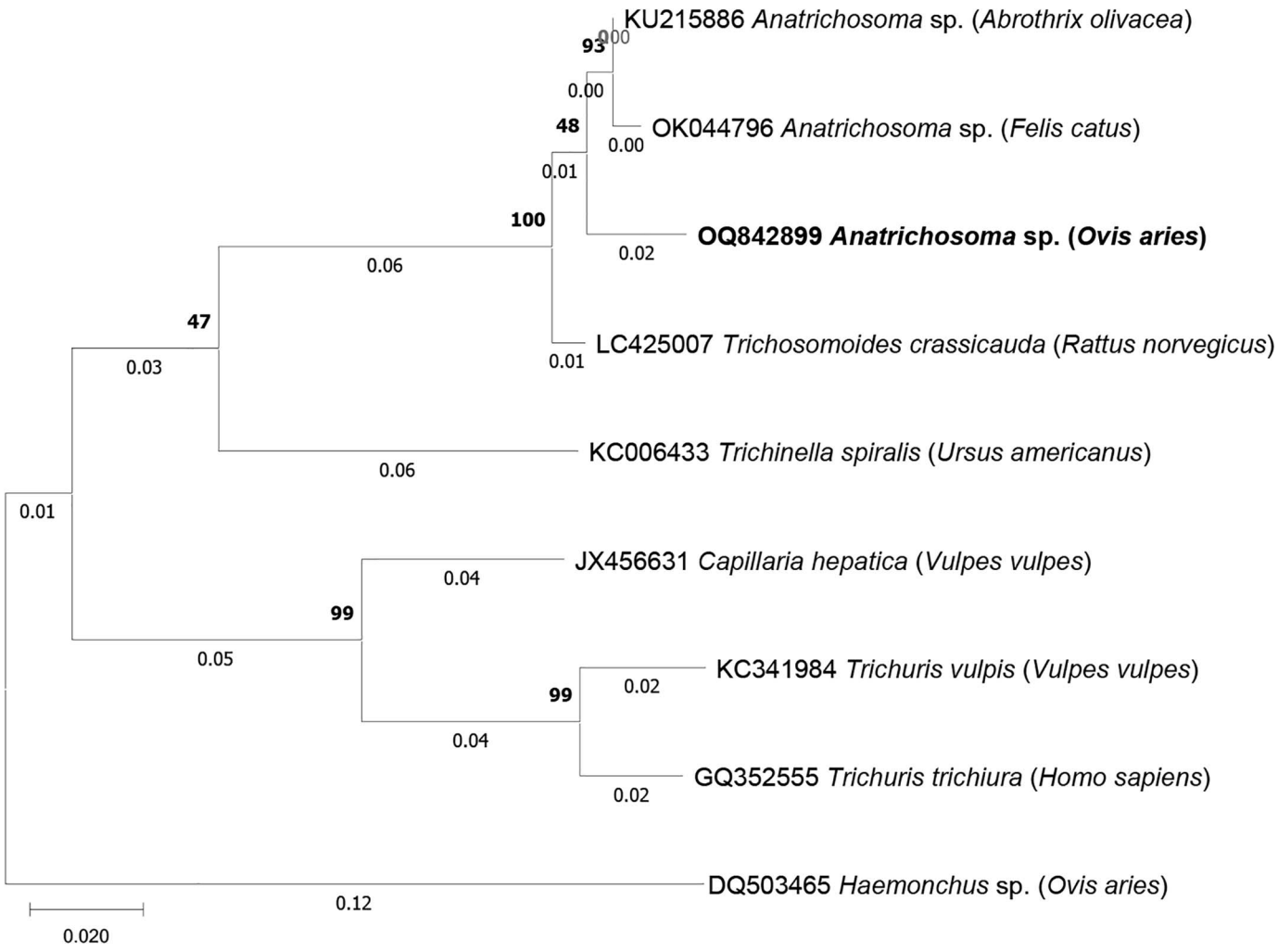

Figure 4. Phylogenetic tree showing the genetic relationship between *Anatrichosoma* sp. in sheep (OQ842899, in bold) and other Trichuroidea nematodes using *18s* ribosomal ribonucleic acid gene, inferred by the maximum likelihood method and the Kimura 2-parameter model. The bootstrap consensus tree was inferred from 1,000 replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths (shown below each branch) measured in the number of substitutions per site. This analysis included 9 nucleotide sequences, with a total of 676 positions in the final data set.

The nematodes were incidentally detected in Sardinian sheep during another study (D'Amico Ricci et al., 2021), which involved splitting open the heads of sheep destined for slaughter along the sagittal axis to isolate *Oestrus ovis* larvae. Therefore, the infection would not have been detected during a routine inspection, as only a visual check was required. Previous studies focusing on *O. ovis* in our study area and other Mediterranean countries revealed no *Anatrichosoma* when the nasal cavities of sheep destined for slaughter were inspected, suggesting that the infection in sheep is sporadic (Caracappa et al., 2000; Dorchies et al., 2000; Scala et al., 2001; Negm-Eldin et al., 2015; Garijo-Toledo et al., 2023). Although it was not possible for the present survey, it would have been interesting to examine the fecal sample of the infected sheep to verify the possible presence of *Anatrichosoma* eggs. In fact, as described in NHPs (Orihel, 1970), eggs grouped in tunnels formed by adult females are shed in the lumen during the process of epithelial regeneration. Eggs can be ingested and then excreted with feces; however, since the prevalence of infection is higher when eggs are detected with nasal swabs compared with fecal examination (Kessler, 1982), it is thought that eggs are more easily expelled through the nostrils (Orihel, 1970).

Because of the poor knowledge of the life cycle of any species in the genus, it was not possible to establish or at least hypothesize the mode of infection for the sheep. Previous reports concerning geographical regions different from the study area have speculated that contact with soil contaminated by eggs excreted by wild hosts, such as opossums or NHPs, is a possible way of transmitting infection by *Anatrichosoma* spp. to domestic animals (Lange et al., 1980; Hendrix et al., 1990). On the contrary, other cases of infection have been reported in domestic animals living in urbanized areas, without any possible contact with wildlife (Noden et al., 2013; Jitsamai et al., 2021).

Similarly, the lack of information on the biology of the parasite makes it difficult to assess its zoonotic potential: in the cases described in the literature, the route of infection for human patients is not known with certainty.

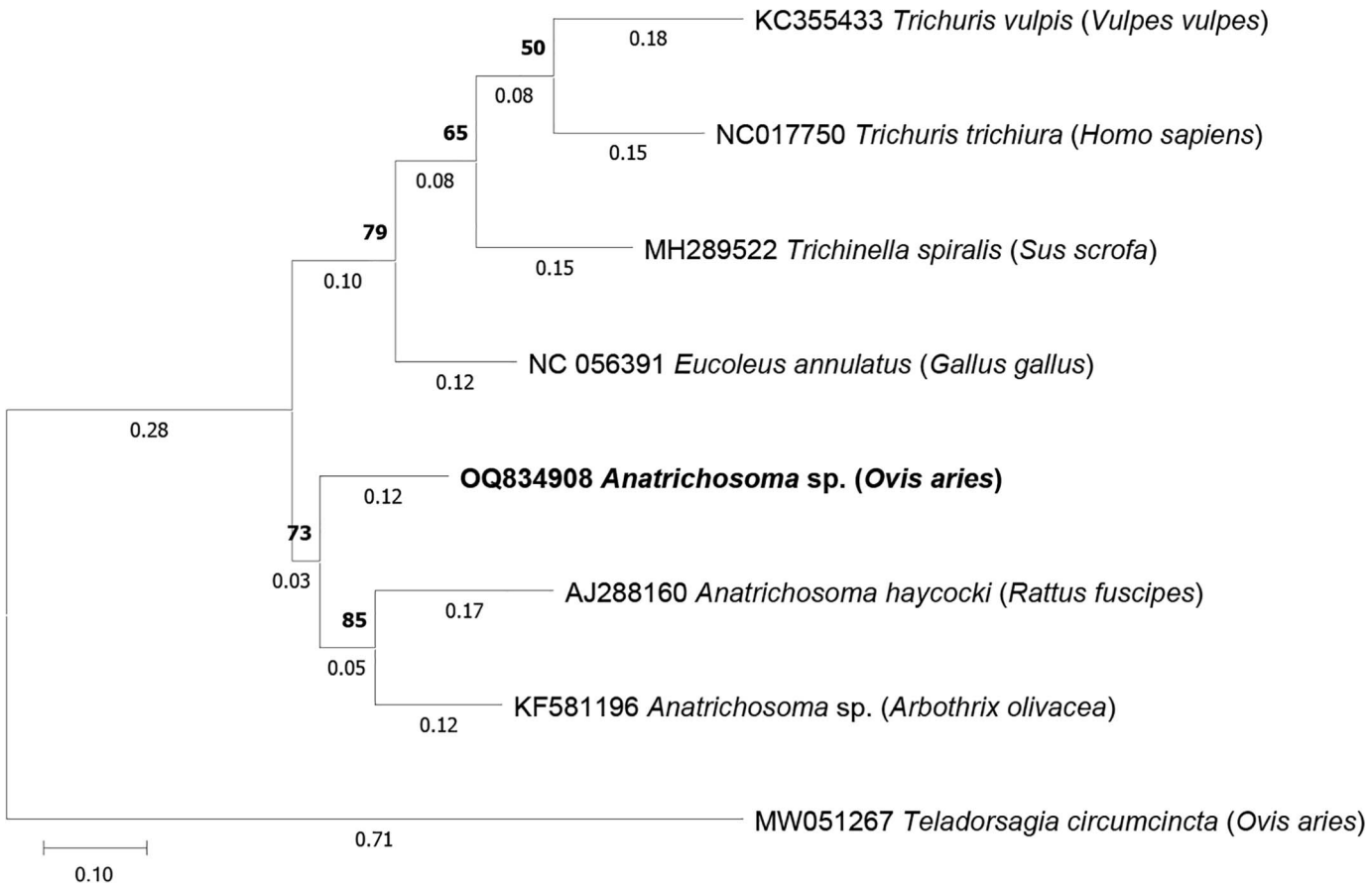

Figure 5. Phylogenetic tree showing the genetic relationship between *Anatrichosoma* sp. in sheep (OQ834908, in bold) and other Trichuroidea nematodes using the mitochondrial cytochrome *c* oxidase subunit I gene, inferred by the maximum likelihood method and the Kimura 2-parameter model. The bootstrap consensus tree was inferred from 1,000 replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths (shown below each branch) measured in the number of substitutions per site. This analysis included 8 nucleotide sequences, with a total of 410 positions in the final data set.

It is interesting to note that a case of infection by *Anatrichosoma* sp. in a human patient had already been described in Italy (Pampiglione et al., 2005). A case of coinfection with *Dirofilaria repens* was diagnosed in a subcutaneous mammary nodule in a woman living in northern Italy with no history of travel abroad. Therefore, it can be speculated that the parasite is present in the Italian territory, although with the available data, it is not possible to conclude whether it is the same *Anatrichosoma* species. In any case, because of the resulting difficulty in detecting the parasite, the diagnosed and reported cases of infection in both humans and animals are probably underestimated.

In addition, the morphological features of the eggs may lead to misdiagnosis because of the similarity of *Anatrichosoma* eggs to those of *Capillaria* and *Trichuris* (Ramiro-Ibanez et al., 2002). Generally, *Anatrichosoma* should be included in the differential diagnosis of parasitic infections with cutaneous or mucosal localization in humans and domestic and wild animals (Núñez, 2010).

A large availability of genetic data of *Anatrichosoma* spp. would have helped in reconstructing possible ways of infection for the host and also the biological cycle of the parasite. Phylogenetic analysis highlighted a certain degree of genetic distance between the sequences obtained in the present study and those of *Anatrichosoma* spp. available in GenBank. The sequencing of the partially conserved *18s* rRNA gene confirmed the morphological identification, showing a 98.47% identity with sequences of *Anatrichosoma* sp.; indeed, the 2 sequences deposited in GenBank of *Anatrichosoma* from the olive glass mouse and from the domestic cat were 100% identical between them. A greater genetic distance was depicted among *Anatrichosoma* sequences of the *COI* mtDNA gene, confirming this gene as an optimal marker to detect genetic differences among closely related species, as previously shown for species belonging to the family Trichuridae (Guardone et al., 2013).

CONCLUSIONS

The present study describes an unusual case of *Anatrichosoma* sp. infection in a sheep, thus adding a new suitable host for this rarely diagnosed parasite. This finding confirms, as already suggested (Núñez, 2010), the need to consider *Anatrichosoma* in the cases of parasitic infections in the mucosa of both humans and animals. Especially in cases of unclear or doubtful parasitic infection, the implementation of the diagnosis by molecular techniques should be desirable for a correct identification of this rare parasite.

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