Morphological and molecular identification of *Spirocerca lupi* (Nematoda: Spiruridae) found in the Andean fox (*Lycalopex culpaeus*)

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Abstract Lesions compatible with spirocercosis were found in the esophagus and aorta of an Andean fox from Cuzco, Peru. The esophageal and aortic lesions were 5.5 and 1.5 cm in diameter, respectively. A total of 12 adult nematodes (6 males and 6 females) were collected from the esophageal lesion, and all were identified as *Spirocerca lupi* by morphological and molecular methods. Molecular characterization was performed by analyzing two sources of the *cox1* gene, and the sequences were compared with previous *S. lupi* sequences from other work deposited in GenBank. Analysis of the partial *cox1* gene from *S. lupi* ($n = 3$) showed 2 haplotypes and had 95–99% nucleotide similarity to previously described sequences. Also, molecular analysis showed that *S. lupi* is a very diverse group, due to the genetic variability of the partial sequences of the *cox1* gene of *Spirocerca*. This study is the first to report finding of spirocercosis in the Andean fox.

Keywords *Spirocerca lupi* · Spirocercosis · Spirurid · Nematode · Andean fox · Culpeo fox · *Lycalopex culpaeus* · Cytochrome c oxidase 1 gene

The Culpeo or Andean fox (*Lycalopex culpaeus*), the second largest canid from South America, is distributed

mainly in the highland from southern Colombia to Tierra del Fuego in Argentina, reaching up to 4800 meters above sea level (Jiménez et al. 1995). The Andean fox is a selective animal and its diet includes different species of vertebrates and invertebrates (Corley et al. 1995).

Several studies have shown that the Andean fox act as a definitive host for several parasites, constituting an accidental disseminator of parasites for other species (McCallum and Dobson 1995). Likewise, the Andean fox can act as a host for helminths of veterinary and public health importance, this due to the association with domestic species that share their habitat (Schantz and Lord 1972).

Spirurid nematodes of the genus *Spirocerca* contain species of veterinary importance worldwide, mostly in the tropical and subtropical regions (van der Merwe et al. 2008). Adult stages of this parasite are found in the esophageal region of dogs and other canids causing the infection spirocercosis (van der Merwe et al. 2008). This disease causes a variety of clinical signs such as dyspnea, regurgitation and vomiting (Mazaki-Tovi et al. 2002). Spirocercosis can cause an acute death due to aortic rupture or a chronic infection, which can produce granulomatous lesions and esophageal fibrosarcomas (van der Merwe et al. 2008).

Currently spirocercosis in foxes has not been reported in Peru. The few reports that do exist about *S. lupi* in Peru are mainly from incidental findings in dogs (Zaldivar 1991). Furthermore, parasitological studies performed in foxes from Peru have not reported *Spirocerca* sp. This work presents the first finding of spirocercosis in the Andean fox (*L. culpaeus*).

In October 2016, a female adult Andean fox (*L. culpaeus*) was found dead near to Sicuani-Condorsenca Road, Km 18. This zone is a rural area located in the Canchis province in Cuzco, Peru (14°15'12.57"S, 71°09'56.94"W,

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4273 meters above sea level). During the necropsy, lesions compatible to spirocercosis were found in the distal third of the esophagus (Fig. 1) and inside the aorta. Nematodes were collected and washed in distilled water. Then, parasites were fixed and preserved using 70% ethanol.

For morphological diagnosis, the nematodes were immersed in lactophenol solution (phenol, lactic acid, glycerol and distilled water 1W: 1V: 2V: 1V) to facilitate the observation of internal organs. Measurements and photographs were taken using a 40-Axioskop microscope (Carl Zeiss, Jena, Germany) and Leica IM50 version 4.0 R117 software (Leica Microsystems, Wetzlar, Germany). The measurements are reported in centimeters (cm) and micrometers (μm), and given as a range with averages and standard error (SE) values in parentheses.

DNA was extracted from three nematodes using the Chelex method according to Gomez-Puerta et al. (2016). Partial sequences of the mitochondrial cytochrome c oxidase 1 gene (cox1) were amplified using two primers sets: COLintF and COLintR (Traversa et al. 2007), and JB3 and JB4.5 (Bowles and McManus 1994). These primer sets amplified the sources 272..932 (660-bp) and 750..1170 (420-bp) of the cox1 gene, respectively. The sources were calculated using the complete cox1 gene (1650-bp) of *S. lupi* (GenBank N° KC305876) (Liu et al. 2013). The polymerase chain reaction (PCR) was performed according to Traversa et al. (2007) and Liu et al. (2013). The PCR products were sequenced using an ABI 3100 automated sequencer (Applied Biosystems). The sequences obtained were compared with previous ones using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The MEGA6 software (<http://www.megasoftware.net/>) was used to create a phylogenetic tree via the neighbor-joining method with Kimura two-parameter distance (Tamura et al. 2004).

Unique nucleotide sequences of the partial cox1 gene of *Spirocerca* sp. from this study were deposited into the GenBank database under accession numbers KY634868, KY634869, KY634870, MF402999, MF403000 and MF403001. This study was approved by the Ethics Committee for Animal Welfare of the School of Veterinary Medicine, University of San Marcos, Lima, Peru.

Pathological lesions of spirocercosis were observed in the aorta and the distal region of the esophagus (Fig. 1a). The aortic wall was thickened at approximately .5 cm and an aneurysm up to 1.0 cm in diameter was noted. The esophageal lesion was irregular in form with a nodular mass of approximately 5.5 cm in diameter. A total of 12 adult nematodes (6 males and 6 females) were collected from the nodule found in the distal region of the esophagus (Fig. 1b).

A total of five male nematodes were analyzed morphologically. The male nematodes measured 3.2–4.6 cm (4.0; SE = .2) in total body length and 670–782 μm (726; SE = 17.0) in maximum body width. The stoma diameter was 85–93 μm (89; SE = 1.4). The buccal capsule measured 68–80 μm (75; SE = 1.9) long and 59–70 μm (64; SE = 2.0) wide. The total length of the esophagus was 5637–6167 μm (5953; SE = 99.5). The muscular esophagus measured 508–544 μm (528; SE = 6.1) long and 121–143 μm (135; SE = 3.6) wide. The glandular esophagus measured 5129–5623 μm (5424; SE = 93.5) long and 309–376 μm (343; SE = 11.3) wide. The distance of the nerve ring, deirids and excretory pore from the proximal end were 497–522 μm (511; SE = 3.9), 499–529 μm (514; SE = 4.7) and 499–529 μm (514; SE = 4.7), respectively. The paired spicules were unequal in size with the left one being longer and thinner, and mildly sclerotized. The greater spicule (left) measured 2359–2977 μm (2698;

Fig. 1 Lesions of spirocercosis in the Andean fox. **a** Esophageal granuloma caused by *Spirocerca* sp. Scale bar = 10.0 cm. **b** Adult *Spirocerca* sp. (forceps) extending through opening in granuloma

SE = 92.4) long and the minor spicule (right) was 639–753 μm (697; SE = 15.3) long. The distance of the cloaca from the distal end was 491–543 μm (521; SE = 9.5).

A total of four female nematodes were analyzed morphologically. The female nematodes were 5.8–7.8 cm (6.5; SE = .3) in total body length and 836–987 μm (908; SE = 20.3) in maximum width. The stoma diameter was 97–105 μm (102; SE = 1.2). The buccal capsule measured 96–111 μm (102; SE = 2.2) long and 59–69 μm (65; SE = 1.5) wide. The total length of the esophagus was 6591–7154 μm (6980; SE = 84.1). The muscular esophagus measured 512–548 μm (531; SE = 5.0) long and 128–142 μm (137; SE = 2.2) wide. The glandular esophagus measured 6078–6605 μm (6449; SE = 79.6) long and 411–465 μm (439; SE = 8.8) wide. The distance of the nerve ring, deirids and excretory pore from the proximal end were 492–540 μm (527; SE = 7.2), 682–702 μm (694; SE = 3.3) and 499–529 μm (514; SE = 4.7), respectively. The vulva opening was located 4009–4386 μm (4224; SE = 54.1) from the distal end. The distance of the anus opening from distal end was 399–429 μm (418; SE = 4.1). The eggs were elongated, embryonated, and measured 31–33 μm (32; SE = .3) long by 13–14 μm (14; SE = .1) wide. The eggs measurements were taken inside the uterus.

Partial sequence of the *cox1* gene was amplified from three *S. lupi* (2 females and 1 male) from the Andean fox. The genetic identity of the worms was calculated from the alignments of the nucleotide sequences. Nucleotide sequences from sources 271..932 of the *S. lupi* *cox1* gene were identical in females (MF402999 and MF403001). The other sequence (MF403000) from *S. lupi* male had a single nucleotide difference of .2% between each other. Likewise, nucleotide sequences from sources 750..1170 were identical in females (KY634868 and KY634870), and they had a single nucleotide difference of .3% compared with the male (KY634869).

These sequences were compared with some previously published *Spirocerca* sequences from Africa, Asia and Europe (Liu et al. 2013, de Waal et al. 2012, Traversa et al. 2007). Regarding nucleotide sequences from sources 272..932 (Traversa et al. 2007) of the *cox1* gene, sequences from this study had less than 5% difference between them. On the other hand, nucleotide sequences from the sources 750..1170 (de Waal et al. 2012) had 1–5% difference between them. The phylogenetic analysis in long sequences (sources 272..932) showed that the Peruvian *Spirocerca* isolates are closer to the isolates from China (Liu et al. 2013) than the isolates from Austria, Italy, Iran, Israel or South Africa (Traversa et al. 2007) (Fig. 2a). In addition, the Peruvian *Spirocerca* isolates form a group with the other isolates except with *Spirocerca* sp. from Danish (Fig. 2a). Therefore, the analysis suggests that the Peruvian

isolates are actually *S. lupi*. This can be confirmed with the analysis obtained in the shorter sequences (sources 750..1170) (Fig. 2b).

The nematodes from this study were identified as *S. lupi* through molecular and morphologic diagnosis. All anatomical measurements and parameters from our specimens were compatible with *S. lupi* (Table 1) (Segovia et al. 2001, Vicente et al. 1997, Skrjabin et al. 1967). Species of *Spirocerca*, mainly *S. lupi*, have been reported in many wild carnivore species worldwide (Skryabin 1991). In South America, *S. lupi* was found in domestic dogs from Brazil, Peru and Venezuela, in a maned wolf (*Chrysocyon brachyurus*) from Brazil, in a crab-eating fox (*Cerdocyon thous*) from Brazil and Venezuela, in a bush dog (*Speothos venaticus*) from Argentina, and in an imported jaguar (*Panthera onca*) from South America kept at the San Diego Zoo (Blume et al. 2014, Santos et al. 2012, Rinas et al. 2009, Vicente et al. 1997, Zaldivar 1991, Bailey 1959, Diaz-Ungria 1956). This finding is the first record of *S. lupi* in the Andean fox.

There are only a few studies about the molecular characterization of the *cox1* gene of *Spirocerca* nematodes. Traversa et al. (2007) examined sources 272..932 of the *cox1* gene of *S. lupi* collected in five countries, and found low genetic variation with all the sequences exhibiting 99% nucleotide similarity. Comparing the sequences of this study with those of Traversa et al. there was 95% nucleotide similarity. Interestingly, de Waal et al. (2012) found high genetic diversity in *Spirocerca* isolates collected from a single dog source. They performed only partial molecular characterization of *S. lupi* analyzing sources 750..1170 of the *cox1* gene. These studies show that *Spirocerca* is a very diverse group, due to the studies carried out with worms from different countries and worms collected from the same host (de Waal et al. 2012; Traversa et al. 2007). On the other hand, Al-Sabi et al. (2014) performed a molecular study in adult worms of *Spirocerca* sp. collected from red foxes from Denmark and found 7–9% of genetic variation between fox's worms and *S. lupi* from Europe, Asia, and Africa. Similarly, our specimens had 8% genetic difference with the *Spirocerca* sp. specimens from the red fox (Al-Sabi et al. 2014). Partial molecular characterization of the *cox1* gene sequences from *S. lupi* generated in our study showed between 95 and 99% nucleotide similarities with previously published *S. lupi* sequences.

The lesions caused by *Spirocerca* in dogs and other definitive hosts are mainly due to the migration and presence of the worms (larvae and adult stages) in the tissue (Mazaki-Tovi et al. 2002). The most frequently noted lesions in dogs are esophageal granulomas and nodular masses, as well as aortic lesions and aneurysms (van der Merwe et al. 2008). However, visceral *Spirocerca* lesions in wild carnivores are more frequent in the aorta than the

A

Phylogenetic tree A shows relationships between *S. lupi* and *Spirocerca* sp. samples. The tree is rooted on the left and branches to the right. Bootstrap values are shown at the nodes. The scale bar represents 0.01 substitutions per site.

Key samples and their locations (indicated by red text):

- S. lupi* (EF394612) - South Africa
- S. lupi* (EF394613) - South Africa
- S. lupi* (EF394611) - South Africa
- S. lupi* (EF394610) - South Africa
- S. lupi* (EF394609) - Iran
- S. lupi* (EF394608) - Iran
- S. lupi* (EF394607) - Iran
- S. lupi* (EF394606) - Israel
- S. lupi* (EF394604) - Israel
- S. lupi* (EF394596) - Israel
- S. lupi* (EF394597) - Israel
- S. lupi* (EF394598) - Israel
- S. lupi* (EF195133) - Israel
- S. lupi* (EF394605) - Austria
- S. lupi* (EF195132) - Italy
- S. lupi* (EF394599) - Italy
- S. lupi* (EF394600) - Italy
- S. lupi* (EF394601) - Italy
- S. lupi* (EF394602) - Italy
- S. lupi* (EF394603) - Italy
- S. lupi* (MF402999) - Peru
- S. lupi* (MF403001) - Peru
- S. lupi* (MF403000) - Peru
- S. lupi* (NC_021135) - China
- S. lupi* (KC305876) - China
- Spirocerca* sp. (KJ605486) - Denmark
- Spirocerca* sp. (KJ605489) - Denmark
- Spirocerca* sp. (KJ605494) - Denmark
- Spirocerca* sp. (KJ605484) - Denmark
- Spirocerca* sp. (KJ605485) - Denmark
- Spirocerca* sp. (KJ605487) - Denmark
- Spirocerca* sp. (KJ605488) - Denmark
- Spirocerca* sp. (KJ605490) - Denmark
- Spirocerca* sp. (KJ605491) - Denmark
- Spirocerca* sp. (KJ605492) - Denmark
- Spirocerca* sp. (KJ605493) - Denmark
- Spirocerca* sp. (KJ605495) - Denmark
- Spirocerca* sp. (KJ605496) - Denmark

B

Phylogenetic tree B shows relationships between *S. lupi* samples. The tree is rooted on the left and branches to the right. Bootstrap values are shown at the nodes. The scale bar represents 0.005 substitutions per site.

Key samples and their locations (indicated by red text):

- S. lupi* (HQ674756) - South Africa
- S. lupi* (HQ674759) - South Africa
- S. lupi* (HQ674753) - South Africa
- S. lupi* (HQ674752) - South Africa
- S. lupi* (KY634869) - Peru
- S. lupi* (KY634868) - Peru
- S. lupi* (KY634870) - Peru
- S. lupi* (HQ674760) - South Africa
- S. lupi* (KC305876) - China
- S. lupi* (NC_021135) - China
- S. lupi* (HQ674754) - South Africa
- S. lupi* (HQ674757) - South Africa
- S. lupi* (HQ674755) - South Africa
- S. lupi* (HQ674758) - South Africa
- S. lupi* (HQ674761) - South Africa

In this study, 2 haplotypes from three sequences were identified in both sources. Similar results were obtained by Traversa et al. (2007) who analyzed sources 272..932 in 20 nematodes collected in Africa, Asia and Europe (Traversa et al. 2007), and Al-Sabi et al. (2014) who analyzed 23 nematodes from red foxes from Denmark (Al-Sabi et al. 2014). On the other hand, de Waal et al. (2012) identified 11 haplotypes analyzing sources 750..1170 in sixty worms collected in South Africa (de Waal et al. 2012). However, as the sample size of *S. lupi* in this study is low (n = 3), the results should be analyzed with discretion. Also, due to the incongruent results obtained analyzing sources 272..932

Table 1 Measurements of *Spirocerca lupi* (in μm) isolated from the Andean fox (*Lycalopex culpaeus*) from the Peruvian Andes

Host	<i>Spirocerca lupi</i>											
	<i>Lycalopex culpaeus</i>			<i>Vulpes vulpes</i>			<i>Canis familiaris</i>			–		
	Present study			Segovia et al. (2001)			Vicente et al. (1997)			Skrjabin et al. (1967)		
According to	n	Range	Mean	n	Range	Mean	n	Range	Mean	n	Range	Mean
<i>Adult male</i>												
Total body length (cm)	8	3.2–4.6	4.0	24	2.52–5.14	4.0	–	3.0–4.0	–	–	3.0–5.4	–
Maximum body width	8	670–782	726	24	608.8–959.7	769.1	–	760	–	–	760–992	–
Nerve ring	8	497–522	511	24	308.6–681.1	464.0	–	–	–	–	285–368	–
Deirids (cervical papillae)	8	499–529	514	24	321.4–743.0	520.3	–	–	–	–	–	–
Muscular esophagus length	8	508–544	528	24	362.6–639.8	523.9	–	–	–	–	520–662	–
Glandular esophagus length	8	5129–5623	5424	24	3100–5900	4400.0	–	–	–	–	4242–8602	–
Esophagus length	8	5637–6167	5953	24	3500–6400	4900.0	–	5800	–	–	–	–
Spicule minor (right)	8	639–753	697	24	596.6–753.3	643.2	–	750	–	–	610–762	–
Spicule mayor (left)	8	2359–2977	2698	24	2100–3300	2800.0	–	2450	–	–	2450–4910	–

and 750..1170 of the *cox1* gene, it is necessary to choose with caution the source or region of the *cox1* gene of *Spirocerca* to analyze.

It is important to take note of the aortic lesions, aneurysms and hemothorax in the fox of this study. The spirocercosis could have been the cause of death, due the fox did not have injuries compatible with traumas or bone fractures. Some studies and case reports have mentioned fatalities caused by spirocercosis in dogs and foxes (Morandi et al. 2014, Gal et al. 2005). Spirocercosis in dogs can cause acute death due to aortic rupture (van der Merwe et al. 2008).

The life cycle of *Spirocerca* involves coprophagous beetles as an intermediate host, as well as birds, lizards, and small mammals as paratenic hosts (van der Merwe et al. 2008), Krahwinkel and McCue 1967). These animals are included in the common diet of the Andean fox (Guzmán-Sandoval et al. 2007), thus facilitating the propagation and transmission of the parasite. The Andean fox was found dead on the Sicuani-Condorcenca Road located in the Andean mountains. It is very probable that *S. lupi* maintains a wildlife cycle in these areas since it is rare for the foxes to have any interactions with domestic dogs. Epidemiological studies will be necessary to understand the current situation of spirocercosis in the Andean regions.

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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 international, national, and/or institutional guidelines for the care and use of animals were followed.

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