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DISCOVERY OF AN UNDESCRIBED PROTOSTRONGYLID NEMATODE FROM THE ENDANGERED PAMPAS DEER (*OZOTOCEROS BEZOARTICUS CELER*) IN ARGENTINA

Ramon A. Carreno,[1,6] Dina Caporossi,[2] Mario S. Beade,[3] Carolina A. Marull,[4] Marcela M. Uhart,[2,4] David D. Markwardt,[1] and Steven A. Nadler[5]

[1] Department of Zoology, Ohio Wesleyan University, 90 South Henry St., Delaware, Ohio 43015, USA
[2] Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil (7000), Buenos Aires, Argentina
[3] Fundación Vida Silvestre Argentina, General Lavalle (7103), Buenos Aires, Argentina
[4] Global Health Program, Wildlife Conservation Society, Puerto Madryn (9120), Chubut, Argentina
[5] Department of Nematology, University of California, 1 Shields Ave., Davis, California 95616, USA
[6] Corresponding author (email: racarren@owu.edu)

ABSTRACT: Dorsal-spined protostrongylid nematode larvae (Metastrongyloidea: Protostrongylidae) were recovered from the feces of the endangered pampas deer (*Ozotocerus bezoarticus celer*) in Campos del Tuyú Wildlife Reserve, Bahia Samborombón, Argentina. Partial DNA sequences from the large subunit ribosomal RNA (LSU rRNA) gene and from the second internal transcribed spacer region (ITS2) were amplified, cloned, sequenced, and compared to those of other nematodes. Nucleotide alignment and phylogenetic analysis of the sequences indicate that this protostrongylid nematode is most closely related to *Parelaphostrongylus* spp. as inferred from the LSU rRNA sequence analysis. Analysis of the ITS2 spacer indicated that the pampas deer protostrongylid is nested in a clade containing *Parelaphostrongylus* and *Elaphostrongylus* spp. These sequences differed considerably from those of other protostrongylid nematodes, and were most similar to those of *Parelaphostrongylus* spp. and *Elaphostrongylus* spp. in spite of clear variability from both genera. These results suggest that the protostrongylid from pampas deer is an undescribed nematode that likely belongs in the subfamily Elaphostrongylinae.

Key words: ITS2 DNA sequence, LSU rDNA sequence, *Ozotoceros bezoarticus celer*, *Parelaphostrongylus*, Protostrongylidae.

INTRODUCTION

Several species of elaphostrongyline nematodes are known pathogens of cervids and other ruminants (Lankester, 2001). In North America, *Parelaphostrongylus tenuis* (Dougherty, 1945) is highly pathogenic in many ruminants other than its natural host, the white-tailed deer (*Odocoileus virginianus*), and many Eurasian ruminants are susceptible to disease caused by infection with *Elaphostrongylus* spp. (Lankester, 2001; Carreno, 2010). The life cycles of these parasites are well characterized, but little is known of their distribution outside North America and their potential pathogenicity in ruminant populations from other localities.

The cervid populations of Central and South America have not been investigated for protostrongylid nematodes and other parasites to the same extent as cervid populations in other parts of the world. *Parelaphostrongylus tenuis* and several other newly documented parasites were reported for the first time outside of North America from white-tailed deer in Costa Rica (Carreno et al., 2001), but *Parelaphostrongylus* spp. have not been reported in South American host populations. Several South American cervid species have endangered status, yet their parasite fauna is poorly understood. Dorsal-spined larvae typical of most protostrongylid nematodes have been reported from pampas deer (*Ozotoceros bezoarticus celer*) in Argentina (Uhart et al., 2003), but adult worms have not been recovered. The distribution of protostrongylid nematodes in pampas deer and other South American cervids has not been studied further with morphologic or molecular approaches, and adult worms have not been reported from these hosts.

In recent years, certain DNA sequences have been found to be reliable for the identification of protostrongylid nematodes. Sequences from the second internal

TABLE 1. Pairwise distances between taxa of protostrongylid nematodes based on the second internal transcribed spacer. Values above the diagonal are mean character distances (adjusted for missing data). Values below the diagonal represent total character differences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *Elaphostrongylus rangiferi* AY648408	–	0.00000	0.00000	0.05114	0.11667	0.11831	0.11765	0.13699	0.12877	0.10955	0.11299	0.11732	0.13499	0.12363	0.12329	0.63399	0.65744	0.37612
2. *Elaphostrongylus cervi* AF504026	0	–	0.00000	0.05099	0.11667	0.11831	0.11765	0.13699	0.12877	0.10955	0.11299	0.11732	0.13499	0.12363	0.12329	0.63518	0.65862	0.37313
3. *Elaphostrongylus rangiferi* AF504033	0	0	–	0.05099	0.11634	0.11798	0.11732	0.13661	0.12842	0.10924	0.11268	0.11699	0.13462	0.12329	0.12295	0.63518	0.65862	0.37500
4. *Elaphostrongylus alces* AF504034	18	18	18	–	0.14159	0.14371	0.14627	0.16422	0.15543	0.13554	0.13253	0.14671	0.15976	0.15000	0.14956	0.63830	0.66045	0.36129
5. *Ozotoceros* isolate JN092130	42	42	42	48	–	0.03601	0.04155	0.05464	0.04645	0.03911	0.04190	0.04722	0.05234	0.04110	0.04098	0.62590	0.64639	0.36957
6. *Parelaphostrongylus andersoni* AY648400	42	42	42	48	13	–	0.01105	0.02479	0.01653	0.00281	0.00562	0.01676	0.02222	0.01105	0.01102	0.63636	0.66538	0.37931
7. *Parelaphostrongylus tenuis* AF504035	42	42	42	49	15	4	–	0.03014	0.02192	0.01117	0.01401	0.00556	0.02762	0.01648	0.01644	0.63538	0.66412	0.37695
8. *Parelaphostrongylus odocoilei* AY648403	50	50	50	56	20	9	11	–	0.01340	0.02473	0.02762	0.03279	0.01892	0.01344	0.01340	0.62807	0.65799	0.36778
9. *Parelaphostrongylus odocoilei* AY648380	47	47	47	53	17	6	8	5	–	0.01648	0.01934	0.02459	0.00541	0.00538	0.00536	0.62807	0.65799	0.37386

Table 1. Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
10. *Parelaphostrongylus andersoni* AF504030	39	39	39	45	14	1	4	9	6	–	0.00276	0.01648	0.02216	0.01099	0.01099	0.61957	0.65385	0.37461
11. *Parelaphostrongylus andersoni* AF504036	40	40	40	44	15	2	510	7		1	–	0.01934	0.02507	0.01381	0.01381	0.61314	0.65504	0.37383
12. *Parelaphostrongylus tenuis* AF504029	42	42	42	49	17	6	212	9		6	7	–	0.03030	0.01918	0.01913	0.61871	0.66412	0.37231
13. *Parelaphostrongylus odocoilei* AY648392	49	49	49	54	19	810	7	2		8	9	11	–	0.01084	0.01081	0.62766	0.65414	0.36810
14. *Parelaphostrongylus odocoilei* AF504031	45	45	45	51	15	4	6	5	2	4	5	7	4	–	0.00000	0.63028	0.66045	0.37195
15. *Parelaphostrongylus odocoilei* AF504037	45	45	45	51	15	4	6	5	2	4	5	7	4	0	–	0.62937	0.65926	0.37576
16. *Muellerius capillaris* AY679528	194	195	195	180	174	175	176	179	179	171	168	172	177	179	180	–	0.29582	0.68478
17. *Umingmakstrongylus pallikuukensis* AY648409	190	191	191	177	170	173	174	177	177	170	169	174	174	177	178	92	–	0.69084
18. *Varestrongylus alpenae* AY648407	126	125	126	112	119	121	121	121	123	121	120	121	120	122	124	189	181	–

transcribed spacer (ITS2) are useful for distinguishing among species of *Parelaphostrongylus* and *Elaphostrongylus* and have been used to determine the distribution of these parasites in North American hosts as well as to determine the identity of other protostrongylids based on DNA acquired from first-stage larvae (Gajadhar et al., 2000; Jenkins et al., 2005; Kutz et al., 2007). Our objective was to use two genetic loci from the nuclear ribosomal RNA repeat to compare DNA sequences acquired from dorsal-spined larvae in pampas deer to those of other known protostrongylids.

MATERIALS AND METHODS

Collection of larvae, DNA extraction, polymerase chain reaction (PCR), and cloning

Feces from pampas deer were collected in November 2006 at Campos del Tuyú Wildlife Reserve, Bahia Samborombón, Argentina (36°15′S, 56°55′W) and neighboring ranches. Feces were collected seasonally (intervals throughout fall, winter, spring, and summer, 2006 and winter, 2007). The collection areas were scouted until *O. b. celer* were found. The deer were observed from a distance using binoculars until an animal was seen defecating. Forty-six pampas deer fecal samples were collected: nine in fall, 17 in winter, 12 in spring, and eight in summer. Five samples were collected from adults, two from juveniles and the rest were from unknown age categories. The majority from unknown age categories were presumed to belong to adults based on their sizes. In the field, feces were collected and stored in air-tight bags and preserved in a cooler with ice packs until processing at a field laboratory within 2–4 hr (those collected at Campos del Tuyú) or 5–8 hr (those collected at Reserva Provincial Río de Ajó). Larvae were recovered from feces by the traditional Baermann technique using funnels (Baermann, 1917). Seventy percent of *O. b. celer* samples had the unknown protostrongylid larvae, which were present in all seasons in adult and juvenile pampas deer feces. Recovered larvae were stored in 90% ethanol and kept at -20 C from the time of collection until processing for DNA extraction and PCR in late 2007.

Approximately 100 first-stage larvae with a dorsal tail spine typical of many species in the lungworm family Protostrongylidae were pooled for nucleic acid extraction. DNA was extracted from larvae using the Qiagen DNeasy® Tissue Kit (Qiagen Inc., Valencia, California, USA) and the protocol for purification of total DNA from animal tissue. A 950-base-pair fragment of the 5′ end of the large subunit ribosomal DNA (LSU rDNA) containing the D2 and D3 domains was amplified using primers 391 forward (5′- AGCGGAGGAAAAGAAACTAA) and 501 reverse (5′-TCGGAAGGAACCAGCTACTA). The ITS2 region was amplified using primers NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′). PCR reactions of 25 µl consisted of 0.5 µM of each primer, 200 µM of deoxynucleoside triphosphates, and 3 mM of $MgCl_2$. Proofreading polymerase (Finnzymes DyNAzyme EXT, New England Biolabs, Ipswich, Massachusetts, USA) was used for amplification. PCR cycling parameters for the rDNA reactions included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 30 sec, 54 C (LSU) or 58 C (ITS2) for 30 sec, and 72 C for 1 min, followed by a postamplification extension of 72 C for 7 min.

Prior to sequencing, PCR products were cloned into pGEM-T using the pGEM-T vector system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Briefly, PCR products were ligated into the pGEM-5Zf(+) vector that had been linearized with EcoRV and thymidinylated. DNA was extracted from bacterial clones using the QIAprep Spin Miniprep Kit (Qiagen), and this purified plasmid was used as the template for sequencing. Inserts were sequenced from T7 and/or SP6 promoters. Sequencing reactions were performed using BigDye-terminator cycle sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer at the Plant-Microbe Genomics Facility, Ohio State University, Columbus, Ohio, USA. DNA templates were sequenced for both strands.

Sequence analysis

Sequences produced for this study were deposited in GenBank as accession numbers JN092130 (ITS2 sequence) and JN122348 (partial 28S sequence). Completed sequences were aligned with other comparable protostrongylid species sequences from GenBank using Clustal X version 1.53b (Thompson et al., 1997). Phylogenetic analysis of ITS2 and LSU rDNA sequences was performed using PAUP*4.0b10 (Swofford, 1998). Phylogenetic trees were viewed and printed using TreeView (Page, 1996). For the ITS2 analysis, sequences from additional protostrongylid species were used as outgroups. These included (GenBank accession numbers in parentheses) *Varestrongylus alpenae* (AY648407), *Muellerius capillaris* (AY679528,

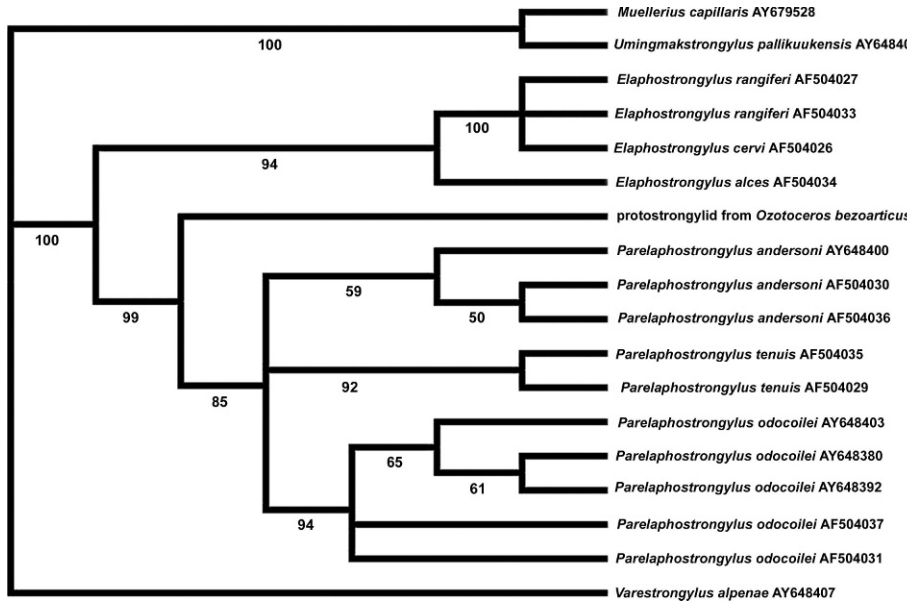

FIGURE 1. Strict consensus of nine equally parsimonious trees depicting phylogenetic relationships among protostrongylid nematodes inferred from second internal transcribed spacer rDNA sequences. Bootstrap values (from 1,000 replicates) for clades exceeding 50% support are shown.

AY679527), and *Umingmakstrongylus pallikuukensis* (AY648409). Several other available ITS2 sequences for *Parelaphostrongylus* spp. and *Elaphostrongylus* spp. from GenBank were used in an alignment (accession numbers shown in Table 1). Sequences used for the LSU rDNA analysis included *Metastrongylus salmi*, which was used as an outgroup, as well as *Protostrongylus boughtoni*, *Protostrongylus rupicaprae*, *U. pallikuukensis*, *P. tenuis*, and *Parelaphostrongylus odocoilei* (AY292803). Sequences were analyzed, and the most parsimonious trees were found using the branch-and-bound search option of PAUP*. Relative reliability of clades was assessed using 1,000 replicates of bootstrap resampling with parsimony inference.

RESULTS

Fresh first-stage dorsal-spined larvae ($n=20$) used in our study averaged 340 μm long and 20 μm wide. In the data set prepared from alignment of the ITS2 rDNA, 106 bases at the 5′ end and positions 558–664 of the alignment were excluded due to missing data. Alignment of ITS2 rDNA showed that the pampas deer isolate differed from all other taxa included in the analysis. A distance matrix for these data reveals greater genetic divergence of the pampas deer isolate from protostrongylids other than *Parelaphostrongylus* and *Elaphostrongylus* species, to which it shows a smaller genetic distance (higher similarity, Table 1). In the alignment used for phylogenetic analysis, there were 183 parsimony-informative characters. Analysis using the branch-and-bound search option yielded nine equally parsimonious trees with a consistency index of 93.6%. In each of these trees, the pampas deer isolate was nested within a clade of elaphostrongyline nematodes and placed as sister to species of *Parelaphostrongylus* (Fig. 1). Parsimony bootstrap support for a sister-group relationship to *Parelaphostrongylus* spp. was very strong (99%).

Sequences from the LSU rDNA gene yielded a similar pattern of high similarity (small genetic distance) between the pampas deer isolate and *Parelaphostrongylus* spp. relative to those of other available protostrongylids (Table 2). The LSU alignment included 95 parsimony-informative characters and yielded a single most parsimonious tree with a consistency index of 90.4%. The pampas deer isolate was the sister taxon to the *Parelaphostrongylus* clade, which includ-

TABLE 2. Pairwise distances between taxa of protostrongylid nematodes based on partial sequences of the large subunit ribosomal (LSU) RNA gene. Values above the diagonal are mean character distances (adjusted for missing data). Values below the diagonal represent total character differences.

	1	2	3	4	5	6	7	8
1. *Protostrongylus boughtoni*	–	0.02303	0.08241	0.07011	0.10732	0.09878	0.10244	0.08680
2. *Protostrongylus rupicaprae*	19	–	0.08487	0.07275	0.10024	0.09291	0.09658	0.07843
3. *Muellerius capillaris* AY292798	67	69	–	0.04645	0.10894	0.10037	0.10404	0.08292
4. *Umingmakstrongylus pallikuukensis*	57	59	38	–	0.09506	0.08889	0.09259	0.06922
5. *Ozotoceros* isolate JN122348	88	82	89	77	–	0.01649	0.02123	0.10192
6. *Parelaphostrongylus tenuis*	81	76	82	72	14	–	0.00707	0.09233
7. *Parelaphostrongylus odocoilei* AY292803	84	79	85	75	18	6	–	0.09592
8. *Metastrongylus salmi* AY292797	71	64	67	56	85	77	80	–

ed *P. tenuis* and *P. odocoilei*. There was 100% bootstrap support for the clade containing the pampas deer isolate and *Parelaphostrongylus* spp. A second clade contained other available protostrongylid taxa including *Protostrongylus* spp., *U. pallikuukensis*, and *M. capillaris* (Fig. 2). There were portions of the LSU rDNA sequences that could not be reliably aligned, including positions 879–901, 1,050–1,064, and 1,233–1,256. A second analysis of LSU data that excluded these three ambiguously aligned regions (86 parsimony-informative characters) yielded similar results to those found in the analysis including all data. Three equally parsimonious trees were found, with relationships similar to those in the first analysis (Fig. 2). These three trees varied in the relative positions of the two *Parelaphostrongylus* spp. and the pampas deer isolate.

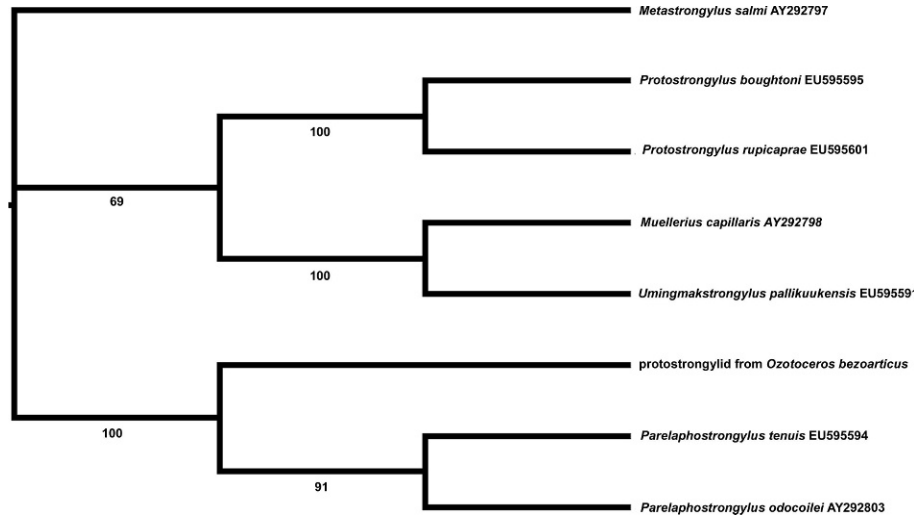

FIGURE 2. Bootstrap 50% majority-rule consensus tree depicting phylogenetic relationships among protostrongylid nematodes inferred from partial 28S rDNA sequences. Bootstrap values (from 1,000 replicates) for clades exceeding 50% support are shown.

Bootstrap analysis of LSU data excluding ambiguously aligned regions provided 100% support for the inclusion of the pampas deer isolate with the two *Parelaphostrongylus* spp.

DISCUSSION

The results from this analysis indicate the presence of an undescribed protostrongylid nematode that is most likely in the subfamily Elaphostrongylinae or very closely related to it. Both the ITS2 (a more variable gene with a higher substitution rate) and the more conserved partial LSU rRNA gene sequence demonstrate this pattern. Comparisons by BLAST database searching of GenBank sequences reveal no other nematodes with higher similarity than the Elaphostrongylinae, and the presence of the characteristic dorsal spine on the tail of the first-stage larva of the pampas deer specimens is also typical of most protostrongylids.

A recent analysis of dorsal-spined larvae from various Arctic ungulates indicated the presence of an undescribed protostrongylid from musk oxen (*Ovibos moschatus*), caribou (*Rangifer tarandus*), and moose (*Alces alces*; Kutz et al., 2007). Analyses using ITS2 sequences indicated that this species is more closely related to *V. alpenae* than to other protostrongylids, and overall larval length and other measurements, while generally smaller than those of several other protostrongylids, overlapped with most other protostrongylids that were measured (Kutz et al., 2007). The measurements of the first-stage larvae taken for this study also overlap with measurements given for other protostrongylids, including other elaphostrongyline nematodes, but are smaller than the length reported for some species with dorsal-spined larvae such as *U. pallikuukensis*, and are slightly larger than previous values reported for *Muellerius capillaris* (Kutz et al., 2007).

Sequences for the ITS2 region have been compiled for several protostrongylids. *Parelaphostrongylus* and *Elaphostrongylus* spp. have been sequenced from several localities. Although slight sequence variation is seen in *P. odocoilei*, the ITS2 can distinguish between all known *Parelaphostrongylus* spp. (Gajadhar et al., 2000; Huby-Chilton et al., 2006; Jenkins et al., 2005). The ITS2 sequences are markedly different between *Parelaphostrongylus* spp. and other protostrongylids (Jenkins et al., 2005), and this result is consistent with several morphologic characters distinct for this genus (Carreno and Lankester, 1993, 1994; Carreno and Hoberg, 1999). Although not all protostrongylid genera are represented by ITS2 sequences in GenBank, the range of taxa used in the present analysis and the sister-group relationship between the pampas deer isolate and *Parelaphostrongylus* spp. strongly support the distinct nature of the Argentine isolate.

The LSU rRNA gene has been shown to be phylogenetically informative in resolving relationships among the Metastrongyloidea (Carreno and Nadler, 2003; Chilton et al., 2006). BLAST similarity, as well as the phylogenetic analysis presented here, provides further support of a close relationship between the pampas deer isolate and *Parelaphostrongylus* spp. based on LSU sequences. In combination with the ITS2 analysis, representative species from each of the main protostrongylid clades identified by Carreno and Hoberg (1999) have been compared to the pampas deer isolate.

It will be necessary to collect adults to determine the precise identity and fully characterize the protostrongylid in pampas deer. Its close phylogenetic affinity to the Elaphostrongylinae suggests that a nonpulmonary predilection site, such as the skeletal muscles or meningeal spaces around the nervous system, is possible. Adult elaphostrongyline nematodes are easily distinguished by morphology (Carreno and Lankester, 1993). Characterization of an additional closely related species has the potential to reveal interesting patterns of evolution for morphologic characteristics and predilection site based on phylogenetic relationships. There is no information on possible gastropod intermediate hosts throughout the range of the pampas deer, and life cycles, pathogenesis,

and overall susceptibility of related cervid hosts to this parasite in South America have not been adequately studied.

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