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MORPHOLOGICAL AND MOLECULAR DESCRIPTION OF *BAYLISASCARIS VENEZUELENSIS*, N. SP. FROM A NATURAL INFECTION IN THE SOUTH AMERICAN SPECTACLED BEAR *TREMARCTOS ORNATUS* CUVIER, 1825 IN VENEZUELA

CARACTERIZACIÓN MORFOLÓGICA Y MOLECULAR DE *BAYLISASCARIS VENEZUELENSIS*, N. SP. DE UNA INFECCIÓN NATURAL EN EL OSO ANDINO DE ANTEOJOS, *TREMARCTOS ORNATUS* CUVIER, 1825 EN VENEZUELA

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ABSTRACT

In this study we report the first description of natural infection with *Baylisascaris* sp. in the South American spectacled bear (*Tremarctos ornatus* Cuvier, 1825) from Venezuela. In November 2010, a spectacled bear was found dead during a routine patrol in The National Park "India Carú" (Bailadores Mérida, Venezuelan Andes). Necropsy revealed congestion, hemorrhage of the lungs and small bowel enlargement. Large amount of big whitish nematodes were found filling the intestinal lumen. Nematodes were initially identified as *Baylisascaris* sp., and they were established as morphologically closely related to *Baylisascaris transfuga* (Rudolphi, 1819), differing in the number of caudal papillae. *Baylisascaris transfuga* has been reported in several species of bears in different countries. Molecular characterization based on analysis of the ITS1, ITS2 rDNA and 5.8S region positioned the spectacled bear specimen in the genus *Baylisascaris*. Furthermore, important divergences were noted when compared to homologous sequences from other members of this genus retrieved from the GenBank. Indeed, several SNPs located into the highly conserved 5.8S as compared with *B. transfuga*, *B. schroederi* (McIntosh, 1939), *B. procyonis* (Stefanski & Zarnowski, 1951) and *B. columnaris* Leidy, 1856, as well as a genealogy network based on complete ITS rDNA sequences (ITS1+5.8S+ITS2) strongly suggest that this specimen constitute a new species of *Baylisascaris*. Taking into account morphological and molecular evidence, the vertebrate host and its particular geographical distribution in South America, we describe our specimens as a new species within this genus and we name it *Baylisascaris venezuelensis*.

Keywords: *Baylisascaris* – ITS2 – ITS1 – 5.8S – *Tremarctos ornatus* – Venezuela

RESUMEN

En este estudio describimos por primera vez un caso de infección natural por *Baylisascaris* sp. en un oso andino de anteojos (*Tremarctos ornatus* Cuvier, 1825) en Venezuela. En noviembre de 2010 un joven oso fue encontrado muerto por los Oficiales de la Guardia Nacional durante un patrullaje rutinario en el parque Nacional India Carú en Bailadores, (Mérida, Andes Venezolanos). La necropsia reveló congestión y hemorragia de los pulmones y agrandamiento del volumen del intestino delgado. Se encontró una gran cantidad de nematodos blanquecinos de gran tamaño llenando el lumen intestinal. Los nematodos fueron morfológicamente identificados como *Baylisascaris* sp. morfológicamente cercano a *Baylisascaris transfuga* (Rudolphi, 1819), difiriendo en el número de papilas caudales. *Baylisascaris transfuga* ha sido registrado en diferentes especies de osos en diferentes países. La caracterización molecular de nuestro espécimen basado en el análisis del ITS1, ITS2 rDNA y la región del 5.8S lo posiciona dentro del género *Baylisascaris.* Adicionalmente, se observaron importantes divergencias cuando se comparó con secuencias homólogas de otros miembros del género obtenidas del GenBank. De hecho, varios SNPs localizados dentro de la región altamente conservada 5.8S al compararlo con *B. transfuga*, *B. schroederi* (McIntosh, 1939), *B. procyonis* (Stefanski & Zarnowski, 1951) y *B. columnaris* Leidy, 1856, así como la genealogía basada en la secuencia completa del ITS rDNA (ITS1+5.8S+ITS2) sugiere fuertemente que este espécimen constituye una nueva especie de *Baylisascaris*. Tomando en cuenta la evidencia morfológica y molecular, la especie hospedadora y su particular y restringida distribución geográfica en los andes suramericanos, sugerimos se considere a nuestro espécimen una nueva especie con el nombre de *Baylisascaris venezuelensis*.

Palabras clave: *Baylisascaris* - ITS2 - ITS1 - 5.8S - *Tremarctos ornatus* - Venezuela

INTRODUCTION

The South American spectacled bear *Tremarctos ornatus* Cuvier, 1825 is the only species of the family Ursidae inhabiting South America. Its geographical distribution comprises the Andean Mountains of Venezuela, Colombia, Ecuador, Peru and Bolivia, and the northern borderline of Argentina. An outstanding morphological feature is the presence of a kind of mask (white circle hair marks) surrounding eyes. This white mask can vary greatly from one animal to other (Röhl, 1949; Mondolfi, 1971; Goldstein, 1989). Despite the importance of the study of animal diseases for conservation efforts, there is a pronounced lack of knowledge about pathogen diversity and susceptibility in wildlife (MacPhee & Greenwood, 2013). It is generally accepted that in their natural habitat the spectacled bears may be affected by several infectious agents, mainly during the first stages of their life (cubs and juvenile). Moreover, anthropogenic factors leading to a suddenly habitat loss and fragmentation give little time for wildlife to adjust to their new circumstances and may cause severe stress on individual species. In fact, rapid habitat loss is the single most primary cause of endangerment. Therefore, wildlife can be victims of human activities resulting in exposure to novel infections or conditions that may affect their response to infections they already harbour (Thompson, 2013).

Diseases caused by parasites are among the more important factors leading to death of

wildlife (Thompson *et al*., 2010). Among them, species of ascarids are highly prevalent and may severely affect the healthy status of vertebrate hosts. *Baylisascaris* spp. are ascarid nematodes affecting different animals. Sprent (1968), reclassified the ascarid nematodes from bears of the genus *Ascaris* and *Toxascaris* into *Baylisascaris,* a genus reported at that time naturally infecting all bears species except spectacled bears. Then, subfamily Ascaridinae (into family Ascarididae) includes *Baylisascaris, Ascaris, Toxascaris, Parascaris, and Lagochilascaris* (Sprent, 1983; Adamson, 1986).

According with Kazacos (2008), there are eight recognized species within the genus Baylisascaris: *B. procyonis* Stefanski and Zarnowski, 1951 (syn. *Ascaris procyonis* Sprent, 1968), in raccoons; *B. columnaris* Leidy, 1856 in skunks; *B. melis* Gedoelst, 1920 in badgers; *B. devosi* Sprent, 1952 in martens; *B. schroederi* McIntosh, 1939, in the giant panda (*Ailuropoda melanoleuca* David, 1869); *B. tasmaniensis* Sprent, 1970, in Tasmanian devils, quolls and native "cats"; *B. laevis* Leidy, 1856, in marmots and ground squirrels and *B. transfuga* Rudolphi, 1819, affecting bears. Another two species more recently described are, *B. ailuri* for the red panda (*Ailurus fulgens* Cuvier, 1825) (Xie *et al*., 2011), and *B. potosis*, in the kinkajou (*Potos flavus* Schreber, 1774) (Tokiwa *et al*., 2014; Tokiwa *et al*., 2016).

Baylisascaris spp. can cause severe larva migrans syndrome in accidental host. By instance, humans are accidental intermediate hosts for *B. procyonis*. Infection follows the ingestion of *B. procyonis* eggs containing infective second-stage larva. In humans, *B. procyonis* larvae undergo aggressive tissue migration that includes the CNS and eyes. (Gavin *et al*., 2005)

Baylisascaris has been reported in grizzly and black bears in Canada (Choquette *et al*., 1968) and in several states of USA (Rush, 1932; Sprent, 1950; 1951; Rogers, 1975; Rogers & Rogers, 1976; Worley *et al*., 1976). *Baylisascaris transfuga* occurs worldwide in bears (captive or free-ranging bears), and the species has been reported in wild bears from USSR (Oshmarin, 1963), Japan (Okoshi *et al*., 1962), Indonesia, Syria, and Tibet (Bromlei, 1965), and USA (Addison, 1978). It has also been reported in captive bears. (Clark *et al*., 1969). More recently, Foster *et al*. (2004) included the presence of parasites in the American black bear (*Ursus americanus* Pallas, 1780). Parasites have also been reported in the european brown bear *Ursus arctos* Linnaeus, 1758 (De Ambrogi *et al*., 2011; Szczepaniak *et al*., 2012; Visser *et al*., 2015), and polar bear *Ursus maritimus* Phipps, 1774 (Testini *et al.*, 2011). These reported were from diverse countries including Japan, Netherlands and Italy. Morphological features of *Baylisascaris transfuga* include: cervical alae present, finelly striated with oral lips marked by a deep groove, fine dentigerous ridges present, 66 precloacal papillae and presence of *area rugosa* near cloaca (Sprent, 1968; Hartwich, 1989)

The species *Baylisascaris shroederi* is considered the most common helminth found in giant panda and it usually parasitizes the intestines causing intestinal obstruction, inflammation, and death (Zhang *et al*., 2008, Zhang *et al*. 2011; Zhang *et al*., 2012). According with Hu (2001), most giant pandas found dead in the wild have been heavily infected with *B. shroederi.* Finally, eggs of supposed *Baylisascaris* spp. were collected in faeces of captive spectacled bears from several US zoos (Schaul, 2006). Although normally the parasite does not cause major symptoms, heavy infection could cause illness or even death (Testini *et al*., 2011).

Tremarctos ornatus is an endangered species and little is known about the parasites that can affect these precious animals. According with

data taken from captive animals, ascarids are the most common parasites affecting *T. ornatus* (Wolff, 1988; WildPro, 2013; Figueroa & Stucci, 2003). Figueroa (2015) found ascarids ova in 28 fecal samples from free ranging *T. ornatus* in Laquipampa Wildlife Refuge (Peru). The aim of this study was to describe, for the first time, a fatal case of natural infection by *Baylisascaris* sp. in a free-ranging young *T. ornatus* from a natural reserve in the Andean Mountains of Venezuela and to describe the morphological and molecular features of this that we consider a new species of *Baylisascaris*, whose control can contribute to the conservation of the spectacled bear in South America.

MATERIAL AND METHODS

A little female specimen of a spectacled bear (*T. ornatus*) was found dead by National Guard Officers during a patrol in the National Park India Carú, Bailadores town, Rivas Dávila Municipality, Mérida, Venezuela (Coordinates: 8.2562189—71.8254101, mean altitude 1830 meters over sea level). The Mountain region of this park belongs to South American Andes.

Since this is an endangered species protected by law, a local veterinarian was called to perform necropsy of the bear and to certify (if possible) the cause of dead. Necropsy was performed at the Experimental Laboratory of the "Universidad Politécnica Kleber Ramírez, *Campus* Bailadores". The specimen was a little female bear, 89 cm length, 40 cm head diameter, 26 kg of weight, 59 cm height. External exploration did not reveal fracture, trauma, gunshot wounds, blood, or any secretion through natural openings. Internal examination at macroscopic level included: Thoracic cavity: lungs with congestion and hemorrhagic focus. Abdominal cavity: Small bowel enlarged, with several segments of yeyuno-ileon of varicous aspect. Observation of the intestine revealed that varicose aspect was due to a large amount of nematodes which were filling the intestinal lumen and were visible under serosa (Fig 1). Stomach was also filled with these parasites. At macroscopic examination these nematodes were ascaroid-like, whitish and large in size (10 to more than 20 cms long). The cause of dead was established as a severe parasitism on the basis of bad body condition and the huge amounts of parasites found. Animal & carcasse was conserved for Bailadores Municipality for taxidermal purposes. Only a few specimens of the nematodes (one female and one male) were conserved in 70% alcohol and sent by the Veterinarian to Helminthology Laboratory, Center for Research in Veterinary Parasitology, School of Veterinary Sciences, Universidad Central de Venezuela (Maracay, state of Aragua, Venezuela, 800 km away) for parasitological and molecular characterization.

Parasitological procedures

Two nematodes, a male and female conserved in 70% isopropyl alcohol were received at the Helminthology laboratory. Male length was 10, 2 cm; female length, 25 cm. Parasites were clarified in lactophenol and identification was made based on Sprent (1968), CIH Keys (Chabaud, 1989; Hartwich, 1989), and Mozgovoi (1953). Parasites were studied under a stereomicroscope (Karl Zeiss® Stemi v2000), and trinocular microscope (Nikon® E200) with a digital camera attached. Pictures of male specimen were also taken with a digital image analyzer system (Nikon®) at the Zoology Museum and Institute, "MIZA" (Agronomic Engineering school, UCV), fixed with pins over a paraffin block. A 2 cm fragment from the middle part of the body of the male specimen was placed in ethanol 99% for molecular techniques No sectioning of the parasite was made for morphological description because only two specimens were available. Thus, morphological examination

was limited to those measures which did not imply that procedure (SanMartín *et al*., 1992; Esquivel, 2011).

A complete specimen (female) and two fragments (caudal and cervical ends) of a male were conserved and deposited on Helminthological collection of the Center for Research in Veterinary Parasitology "Dr. Manuel Antonio Rivera Acevedo" (CIPV-MARA), School of Veterinary Sciences, Universidad Central de Venezuela. (Catalogue number: CIPVMARA2010-N-EX-999).

Molecular procedures

Total DNA was extracted from a male parasite by incubation of a small segment of the middle part of the specimen (50 mg) in a lysis buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl, pH 8.0 and 350 mg/ml of proteinase K) at 37 ºC for 18 h. Then, sample was centrifuged at 14000 *g* for 5 min. and DNA was purified using Wizard DNA Purification Systems (Promega®). An aliquot of 1-2 µL was used as template for amplification of the whole ITS rDNA (wITS: ITS1-5.8S-ITS2) using primers and reaction conditions previously described (Gasser *et al.*, 1996). PCR products from three independent reactions were pooled, resolved by agarose gel (1.5%) electrophoresis, stained with ethidium bromide (0.5 µg/ml), excised from the agarose gel, purified with a Spin-X® kit (Costar®), and cloned with a TA-Cloning® kit (Invitrogen®). The sequences of 5 clones were determined by automated sequencing and clones representing the polymorphism detected were selected for genetic analyses. Sequences were aligned using the ClustalX program (Thompson *et al*., 1997) and refined manually.

The availability of wITS rDNA sequences from *Baylisascaris* species at the GenBank is limited and both for this genus and other related ascarid parasites (roundworm) majority of complete ITS rDNA sequences are restricted to ITS1 rDNA; whereas ITS2 rDNA sequences are as partial sequences. Therefore, genealogy and genetic relatedness of the *Baylisascaris* sp. from the Venezuelan spectacled bear was accomplished based on three independent alignments constructed using sequences herein determined and sequences retrieve from the GenBank: Alignment 1: ITS1 rDNA sequences; this alignment was constructed using complete ITS1 rDNA sequences from the new specimen from spectacled bear from Venezuela (three cloned sequences) plus diverse sequences from the main related roundworm parasites retrieved from the GenBank. ITS1 sequence from *Bunostomum phlebotomum* Railliet, 1900 was used as outgroup. The aim of this alignment was to positioning the new sequences from the roundworm from Venezuelan bear in a genealogy including parasites from the genus *Baylisascaris*, *Ascaris*, *Parascaris*, *Raphidascaris*, *Krefftascaris*, *Toxocara*, *Hysterothylacium*, *Contracaecum*, *Anisakis* and *Pseudoterranova*. Accession numbers of sequences retrieved from the GenBank are showed in Figure 2A. Alignment 2: ITS1 rDNA sequences; this alignment was constructed with ITS1 rDNA sequences from exclusively species of the *Baylisascaris* genus. The aim was to assess the relatedness of the new sequences determined in this study with others species of the *Baylisascaris* genus. Alignment 3: ITS2 rDNA sequences; this alignment was also constructed exclusively with sequences (partial or complete ITS2 rDNA) from species of the *Baylisascaris* genus and the aim was to assess the relatedness of species within the *Baylisascaris* genus and to compare with ITS1 analysis. Accession numbers of ITS1 and ITS2 rDNA sequences retrieved from the GenBank are showed in Figure 3A and 3B. *Ascaris summ* ITS1 or ITS2 rDNA sequences retrieved from the GenBank was used as outgroup for alignment 2 and 3, respectively. Finally, an alignment displaying the sequence polymorphism within the conserved region 5.8S rDNA of *Baylisascaris*

species was constructed. The aim was to reflect the single nucleotide polymorphisms (SNPs) in this highly conserved rDNA region that support the new spectacled bear specimen as a new *Baylisascaris* species (Fig. 2B).

DNA sequence analyses

Entire cloned sequences (ITS1+5.8S+ITS2) were initially used as query for sequence identity using the BLAST program (nucleotide blast) at the NCBI Home Page (http://www.ncbi.nlm.nih.gov/) using the blast algorithm with the standard searching setting. Newly obtained sequences were aligned as described above and used for phylogenetic analyses as following:

Alignment 1 was used for construction of the Network genealogy using the Neighbor-Net method with Kimura 2 parameters implemented in Splits Tree4 V4.10 (Huson & Bryant, 2006). Internode support was estimated by performing 100 bootstrap replicates using the same parameters optimized for network inferences. Furthermore, this alignment was used for genealogy reconstruction using Bayesian method. Alignments 2 and 3 were used for genetic relatedness among ITS1 or ITS2 rDNA sequences from *Baylisascaris* species. These sequences were assessed by Bayesian, ML and Maximum Parsimony (MP) methods. Bayesian analysis was done using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Tree searches employed GTR plus gamma and proportion of invariable sites. The first 25% of the trees from 100000 generations were discarded as burn in. ML trees were inferred using RAxML v.7.0.4 (Stamatakis, 2006) using the GTRGAMMA model, gamma shape parameter and proportion of invariable sites, with maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal supports were estimated using 500 replicates using a rapid bootstrapping algorithm. MP and bootstrap analyses were performed using PAUP* v. 4.0b10 (Swofford, 2002) with 100 replicates of random addition sequence, followed by branch swapping (RAS–TBR), as previously described (Li *et al*., 2012; Zhao *et al*., 2012; Tokiwa *et al*., 2015). Distance matrices were produced using the uncorrected *p* distance. The alignments of the ITS rDNA sequences are available from the authors by request.

RESULTS

Morphological identification

Nematodes were identified as Order Ascaridida, superfamily Ascaridoidea based on size (male: 10,5 cm; female: 25 cm) and presence of three characteristic lips at the cephalic end, genus *Baylisascaris* sp., first time reported naturally infecting *T. ornatus* in Venezuela, on the basis of:

1. host
2. denticular ridges on internal surface of lips and a marked groove surrounding lips, with cervical alae conspicuous, slightly curled and striated (Fig. 4, 5)
3. Caudal extremity of male showing equal stout, not too long spicules (0,9 mm) and presence of *area rugosa* near cloaca of male (Fig. 6)
4. Posterior margin rounded, Number of papillae (Fig. 7): as in several species of *Baylisascaris*, there are a high number of pre-cloacal papillae. For our specimen we found 44 pre-cloacal papillae, differing from *B. transfuga*, the specific species of bears, which has 66 (according with Sprent, 1968). Table 1 and Table 2 summarizes the major features of our specimens, being the remarkable difference in the number of precloacal papillae.

Molecular analysis

In this study we evaluated polymorphic sequences from the internal transcribed spacer

(ITS1 and ITS2 rDNA), as well as sequences from the highly conserved 5.8S gene.

Molecular identification and wITS sequence analysis

In this study were sequenced five clones of the entire ITS rDNA (wITS), which spanning the regions of the ITS1+5.8S+ITS2. A BLAST search of wITS sequences here determined showed 90% and 89% sequence identity with *B. transfuga* from *T. maritimus* (Italy) and *B. schroederi* from *Ailuropoda melanoleuca* (China), respectively (accession numbers HM594951 and JN210911-JN210912). This initial BLAST search allowed us to suggest that the spectacled bear parasite belong to the *Baylisascaris* genus. However, the percentage of identity of the new sequences with others species of the *Baylisascaris*, as well as the host and geographical origin of the new specimen suggests a putative new genotype or species within this genus. Therefore, the new sequences were used for genealogy reconstruction and relatedness analysis

The wITS rDNA from the spectacled bear from Venezuela was 927-930 base pair (bp) length, a total of 17-20 bp longer than those from *B. transfuga* (HM594951) and *B. schroederi* (JN210911, JN210912), which displayed sequences of 910 bp. Except for the isolates from *B. transfuga* and *B. schroederi* referred above, no other *Baylisascaris* species has complete ITS1 or ITS2 rDNA sequences for length and sequences comparison at the GenBank.

Three out of five wITS rDNA clones determined from the spectacled bear specimen were polymorphic. However, the polymorphism detected was limited to both length variations in a poly-A region within the ITS1 rDNA and an additional microsatellite repeat (GA) within the ITS2 rDNA. Thus, the divergence among cloned sequences was ~0,1%. ITS1 rDNA sequences spanning nucleotides 1 to 432 (two clones), 434 (two clones) and 435 (one clone). These lengths were different from the shorter sequences of *B. transfuga* and *B. schroederi* (428 bp). Similarly, the new ITS2 rDNA sequences determined (two polymorphic sequences out of the five clones) varied from 336 to 338 bp length, differing from sequences from *B. transfuga* (HM594951) and *B. schroederi* (JN210911, JN210912, EU642816), which were 325 bp length. Variations in the length of poly-A motifs in both ITS1 and ITS2 rDNA and in the number of microsatellite repeats in the ITS2 region were the source for length variations of the new sequences from the Venezuelan bear roundworm when compare to *B. transfuga* and *B. schroederi*. Similarly, an insertion of a simple sequence motif (ATA) in the ITS1 rDNA influenced the length variations. However, several SNPs distributed in both the ITS1 and ITS2 rDNA split *B. transfuga* and *B. schroederi* from the *Baylisascaris* specimen from the spectacled bear from Venezuela.

The more conserved 5.8S rDNA region from the Venezuela specimen was 155 bp length. This length was identical for all *Baylisascaris* species available at the GenBank, including *B. transfuga*, *B. schroederi*, *B. procyonis* and *B. columnaris* (Figure 2B). The 5.8S sequence from *B. potosis* available at the GenBank is a partial sequence precluding an accurate comparison. Two SPNs located in the highly conserved 5.8S region separated the new sequences from all other *Baylisascaris* species, i.e: transitions at positions 27 (T by C) and 104 (A by G) (Fig. 3B).

Analysis of sequence polymorphisms within the wITS rDNA

For analysis of sequence polymorphisms (wITS rDNA) the ambiguously aligned regions were excluded by mean of Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The genetic analysis disclosed significant divergences among *Baylisascaris* sp. from *T. ornatus* from

Venezuela and sequences from other *Baylisascaris* species. The divergence among the five wITS clones of *Baylisascaris* sp. herein determined was small (~0.1% internal divergence). However, high divergences separated these sequences from those from *B. transfuga* (8.2%), *B. schroederi* (9.4%) and, *B. procyonis* (10%). The divergence among *B. transfuga* and *B. schroederi* was 2.8%; while larger divergences separated *B. procyonis* from *B. schroederi* (~8%) and *B. transfuga* (~8%). Sequences from the wITS (ITS1+5.8S+ITS2) from *B. columnaris* and *B. potosis* available at the GenBank were too incomplete for comparative purposes.

Sequence polymorphisms regarding to the 5.8S regions revealed ~2% of divergence among *Baylisascaris* sp. from spectacled bear and all the other 5.8S sequences analyzed (*B. transfuga*, *B. procyonis*, *B. columnaris* and *B. schroederi*). The 5.8S from *B. potosis* (accession number KF680774) was not used for comparison because the first 53 nucleotids are missing. Interesting, three SNPs at the highly conserved 5.8S separate the spectacled bear parasite from *B. transfuga* (from *U. maritimus / U. arctos*) and *B. schroederi* (from *Ailuropoda melanoleuca*), which share the same 5.8S sequences (transitions at positions 27, 104, 114). Similarly, three SNPs split the spectacled bear parasite from *B. columnaris* (from *Mephitis mephitis* Schreber, 1776) and *B. procyonis* (from *Procyon lotor* Linnaeus, 1758), which also share 5.8S sequences (transitions at positions 27, 104, 105). At least one SNP separate *B. potosis* from the new Venezuelan roundworm specimen even when the 5.8S sequence from *B. potosis* is incomplete (Fig. 2B).

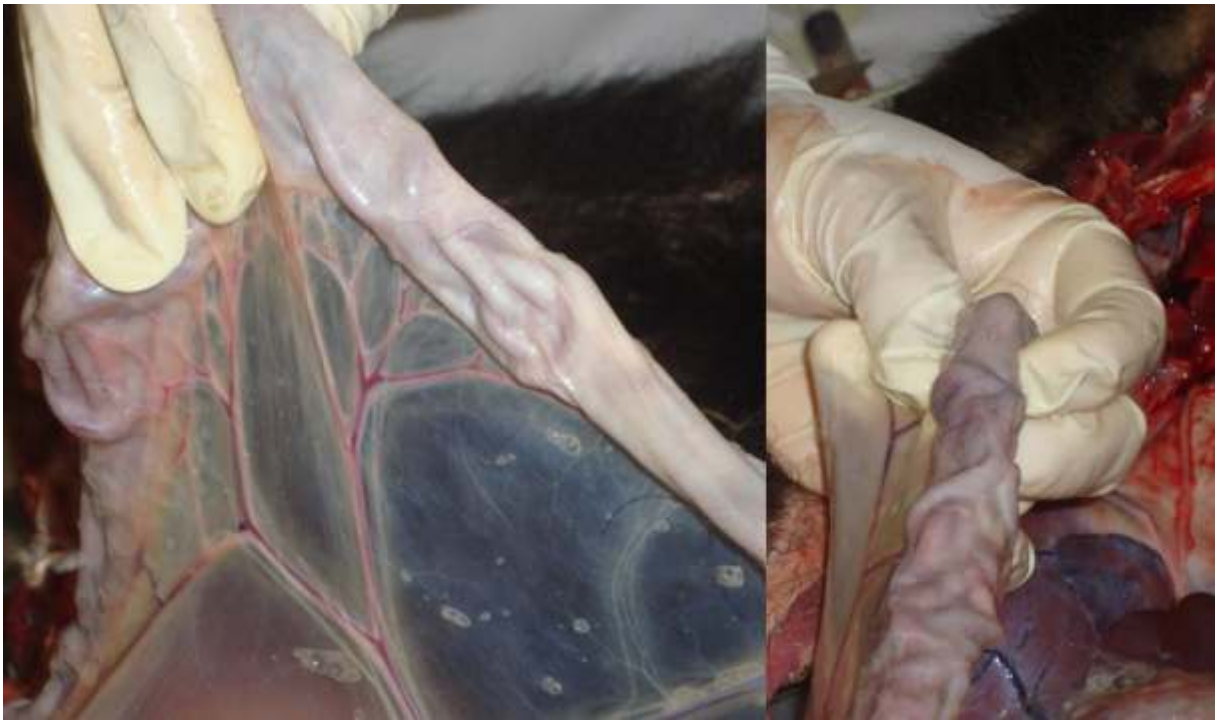

Figure 1. Intestine with varicose aspect due to a large amount of nematodes (*Baylisascaris venezuelensis*) filling the intestinal lumen and visible under serosa.

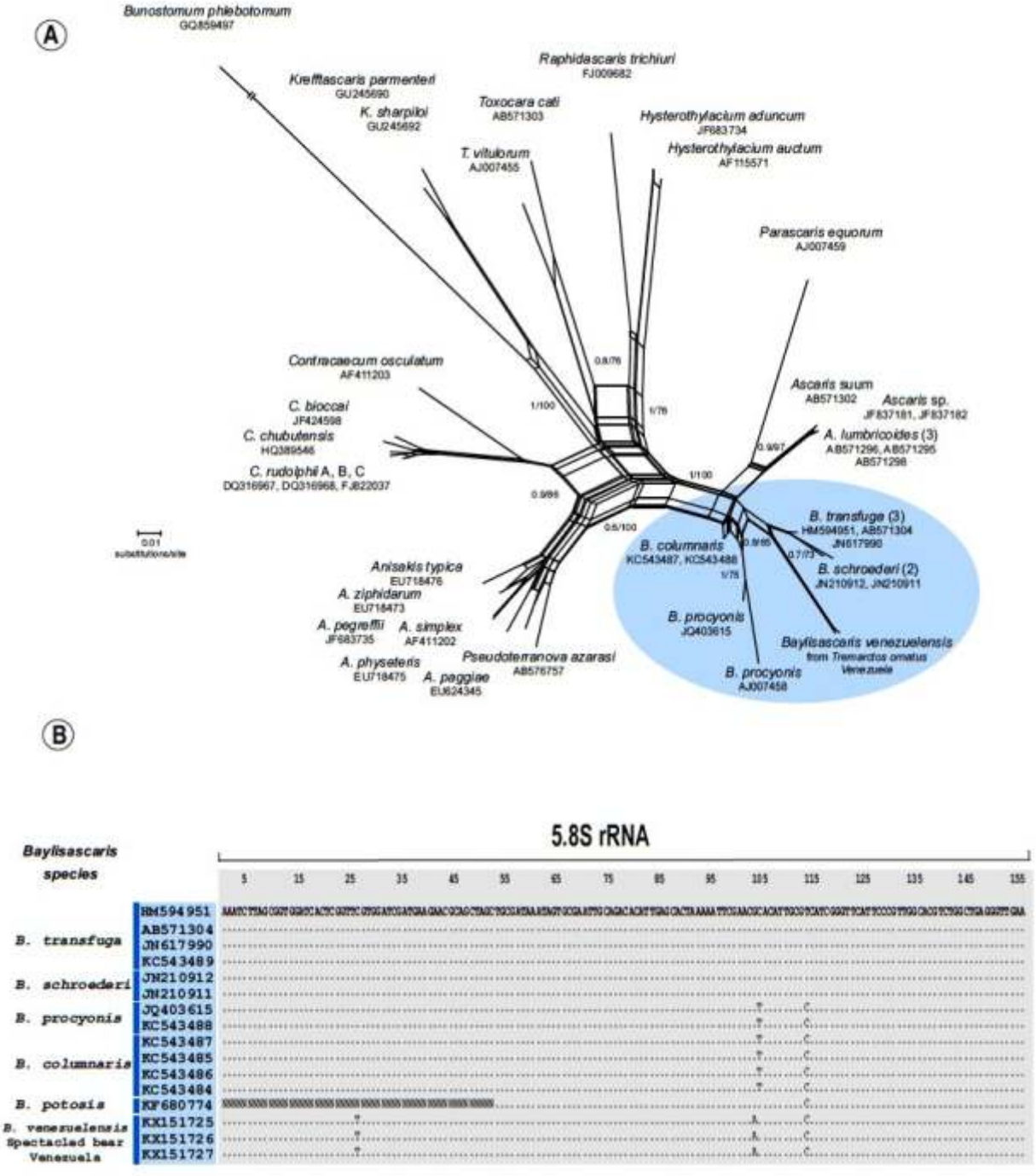

Figure 2. (A). Network genealogy of the ITS-1 rDNA sequences of parasites in the Order Ascaridida showing the position of *Baylisascaris venezuelensis* n. sp., inferred using the Neighbour-Net method with the K2P parameter and Bayesian analysis. *Bunostomum phlebotomum* (GenBank GQ859497) was used as outgroup. Numbers at nodes represent: Posterior probabilities for the Bayesian analysis (first value) and bootstrap values from 100 replicates for the Network analysis (second value). **(B)**. Alignment of the 5.8S rRNA gene sequences from *Baylisascaris venezuelensis* n. sp and comparison with homologues from other *Baylisascaris* species. This figure shows the single nucleotide polymorphisms (SNPs) that split the new *Baylisascaris* species from *Tremarctos ornatus* from Venezuela from the other related *Baylisascaris* spp. Dots represent identical nucleotides, while "N" represent not determined ones.

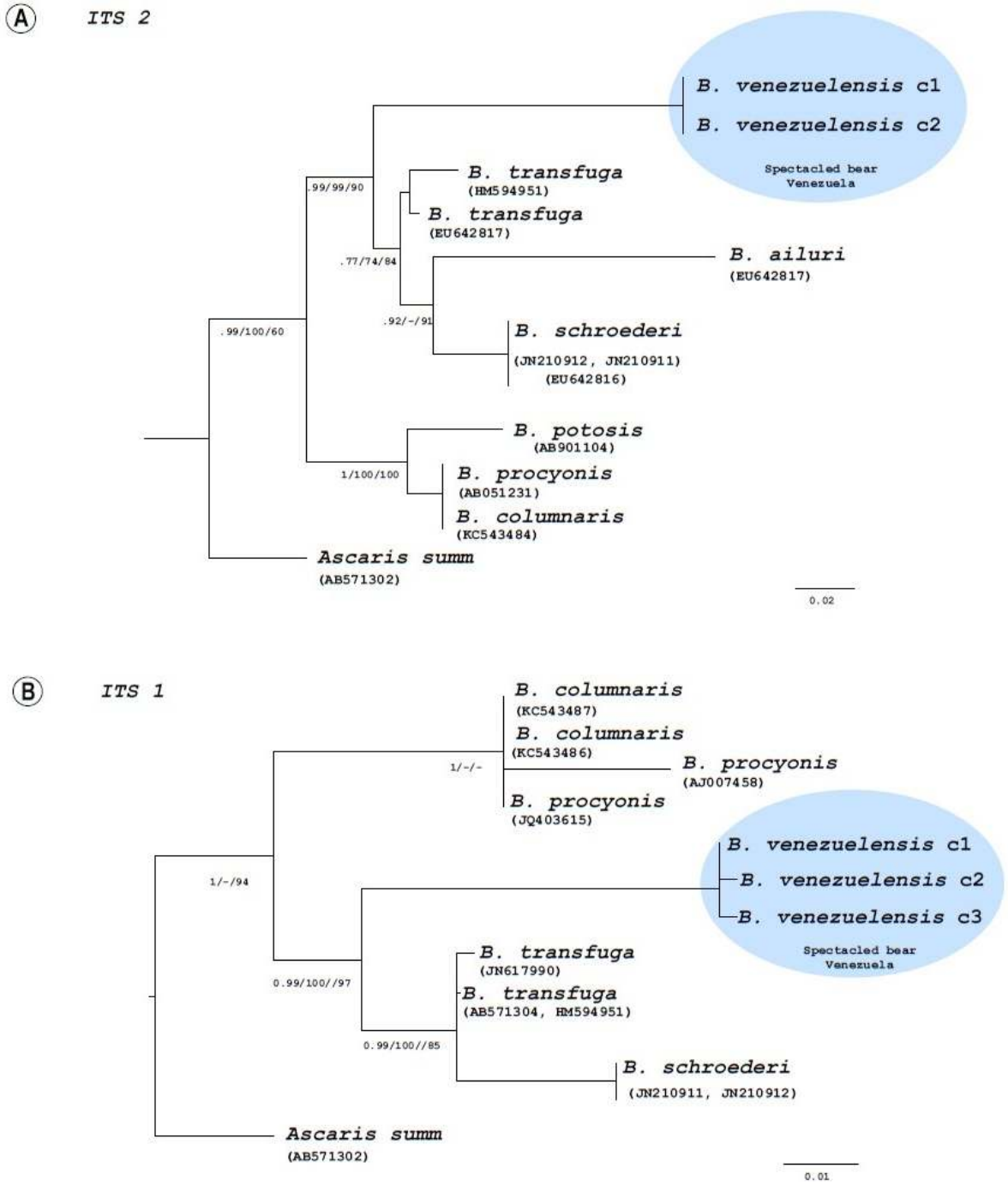

Figure 3. Phylogenetic relationships of *Baylisascaris* species inferred by Bayesian (B), Parsimony (P) and Maximum likelihood (ML) analysis based on ITS-2 rDNA **(A)** and ITS-1 rDNA **(B)** sequences, with *Ascaris summ* (GenBank AB571302) as outgroup. Numbers at nodes represent: Posterior probabilities for the Bayesian analysis (first value), maximum parsimony (second value) and maximum likelihood analyses (third value).

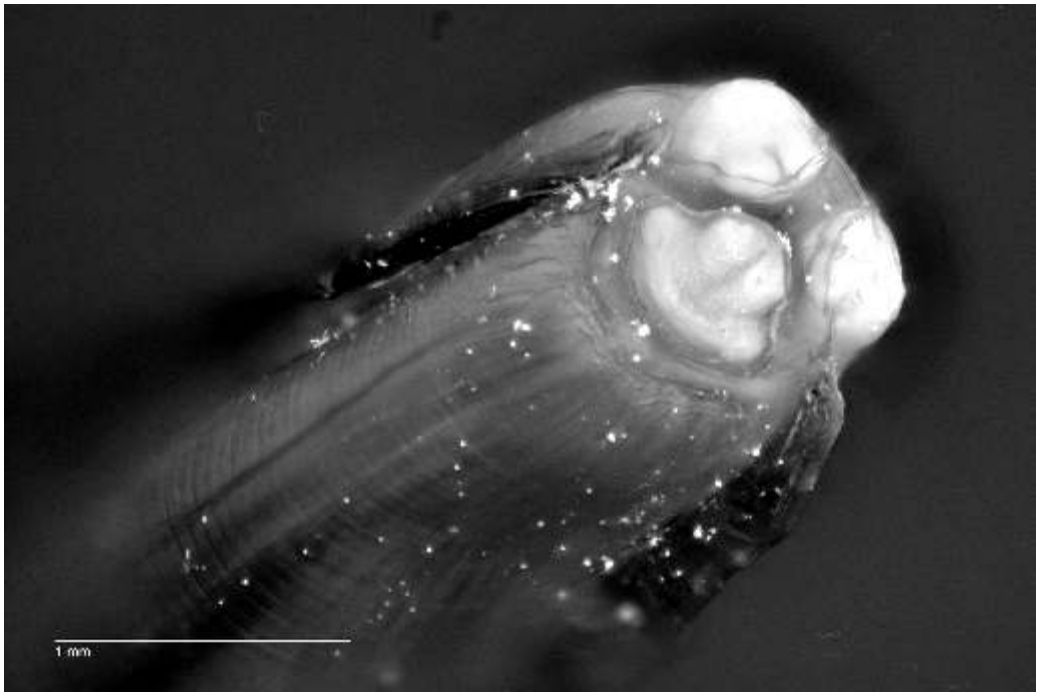

Figure 4. *Baylisascaris* sp. from *T. ornatus* (Venezuela). Head (lateral view). Female.

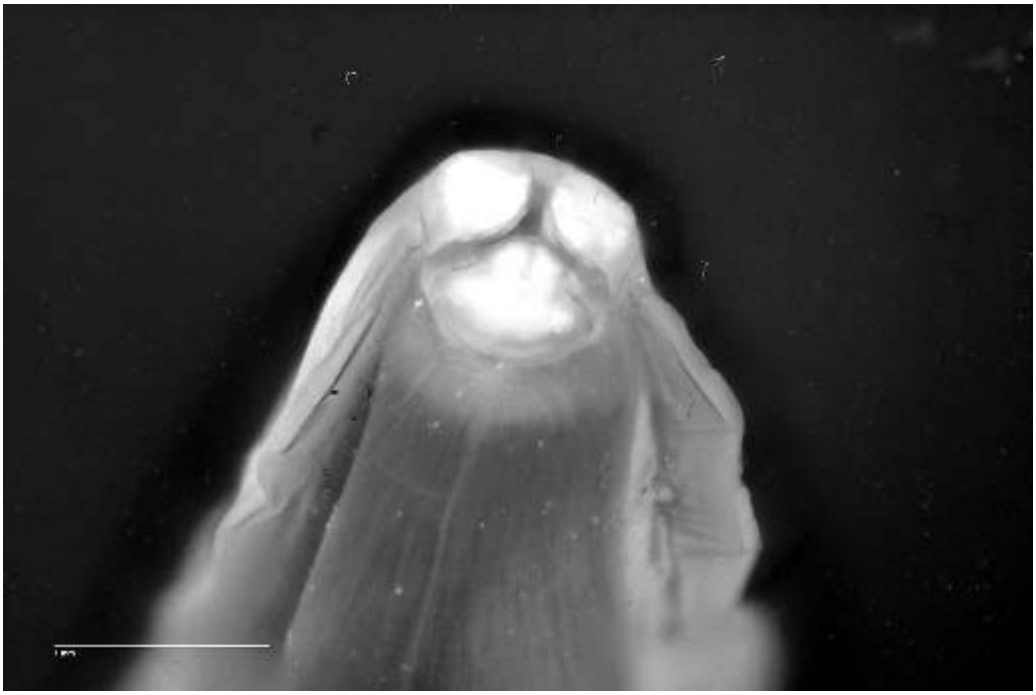

Figure 5. *Baylisascaris* sp. form *T. ornatus* (Venezuela). Head. Detail of groove surrounding lips and dentigerous ridges.

Figure 6. *Baylisascaris* sp. from *T. ornatus* (Venezuela). Male tail showing round end (with a little process) and two short, stout spicules.

Figure 7. *Baylisascaris* sp. from *T. ornatus* (Venezuela). Male tail showing pre-cloacal papillae

Table 1. Some morphological features of *Baylisascaris* sp. infecting *T. ornatus* from Bailadores (Venezuela) compared with six different species of *Baylisascaris*.*

Species	*Baylisascaris sp. (Venezuela)*	*B. transfuga**	*B. melis**	*B. devosi**	B. columnaris*	*B. procyonis**	*B. schroederi**
Male length (cm)	10,2	10,2	12,7	12,3	9,0	9,0	9,8
Female lenght (cm)	25	24,0	22,4	28,5	22,5	20,0	12,5
Alae	Salient, striated	Salient	Salient	Vestigial	Vestigial	Vestigial	Vestigial
Dentícules	Equilateral triangle	Equilateral triangle	Equilateral triangle	High triangles	Equilateral triangle	Equilateral triangle	Inconspicuous
Shape of median lobe of lips	Saddle	saddle	Saddle				
% leght anterior to vulva	40	37	37	33	25	25	34
Female tail (mm)	2	1,4	1,6	1	0,89	0,94	1,4
	Button (round)	Button	Button	Button	Button	Button	Point
Spicules (mm)	0,9	0,93	9,9				
Male tail (mm)	0,5	0,6	0,52				
Pre cloacal papillae (nº)	44	66	63	40	40	43	70
Pre cloacal area	Posterior margin round (with a little process)	Posterior margin round	Posterior margin round	Posterior margin round	Pointed	round	round

*: measures recorded from Sprent (1968).

Table 2. Additional measures recorded from *Baylisascaris* sp. infecting *T. ornatus* from Bailadores (Venezuela).

	Features						
	Lenght (mm)	Width (esophageal región, mm)	Ratio Width / Lenght	Width at anal/cloacal región (mm)	Lenght of tail	Ratio Lenght of tail/Lengh	Ratio Lenght of tail/width at anal region
Male	102	3mm	0,029	0,8	0,5	0,005	0,625
Female	250	4,1mm	0,016	2,1	2	0,008	0,952

DISCUSSION

The nematode genus *Baylisascaris* (order Ascaridida, superfamily Ascaridoidea) contains ten relatively host-specific, parasite species of carnivores, omnivores, herbivores, carnivorous marsupials or rodents (Rogers & Rogers, 2013; Tokiwa *et al.*, 2014). *Baylisascaris transfuga* is considered the specific ascarid of bears, and has been identified naturally infecting all species of bears, excepting spectacled bear (Choquette, *et al.*, 1968; Sprent, 1968; Crum *et al.*, 1978). Reports of the presence of *Baylisascaris transfuga* or undetermined ascarids infecting the spectacled bear was made on the basis of fecal examination of captive individuals (Schaul, 2006, Figueroa, 2015) and Acosta *et al.* (2015) did no found evidence of helminth infection in captive spectacled bears from Zoo "Las Leyendas" (Peru). We had several limitations for the morphological study, i.e. we only had two specimens, and because of these a careful examination of internal structures was not possible, since we need the specimens as holotype and allotype. Even though, the morphological features of *Baylisascaris*

species are particularly externals, and remain in very small details, like number of precloacal papillae, shape of denticles, or shape of tail in posterior margin (species described by Sprent, 1968), or even more difficult, can be distinguished like in *B. potosis* just by the position of the male phasmidial pole (Tokiwa *et al*., 2014). Morphological identification of parasites from wildlife can be a difficult, time-consuming task and usually needs expertise and the aid of DNA analysis (Sepúlveda & Kinsella, 2013). Regarding *Baylisascaris* genus, identification of most recently described species and confirmation of the older ones are highly supported by molecular studies (Zhang *et al*., 2008; Testini *et al*., 2011; Zhang *et al*., 2011; Zhang *et al*., 2012; Zhao *et al*., 2012; Zhou *et al*., 2013; Tokiwa *et al*., 2014; Tokiwa *et al*., 2015; Visser *et al*., 2015). Analyses based on molecular characters rather than morphological ones are needed in the study of these ascarid nematodes phylogeny to evaluate the previous findings. The application of molecular approaches may provide important evidence needed to clarify the taxonomic status and to determine the relationships among these ascarid nematodes (He *et al*., 2013). Although our nematodes primarily appeared to belong to the species *B. transfuga,* a careful examination of the caudal end of male revealed differences with features described for *B. transfuga*. i.e: number of pre-anal papillae. According with Sprent (1968) *T. transfuga* has 66 pre-cloacal papillae while our specimen has 44 papillae. Until the present work no evidence of natural infection with *Baylisascaris* sp. in wild specimens of *T. ornatus* had been reported. Our molecular studies supported the importance of the morphological differences we found between our specimens and *B. transfuga.*

Genealogy and genetic relatedness using ITS rDNA sequences

In this study, a genealogy network analysis considering several related nematode parasites unambiguously positioned the new sequences from Venezuela within the genus *Baylisascaris,* near to ascarid of the genus *Ascaris* and *Parascaris*, which were the sister groups. The network genealogy using ITS1 rDNA sequences from *B. transfuga*, *B. procyonis*, *B. schroederi* and *B. columnaris* from the GenBank, as well as diverse sequences from related parasites grouped the spectacled bear roundworm sequences together with all *Baylisascaris* sequences analyzed in a monophyletic assemble, the genus *Baylisascaris*. Regardless of the analytical approach (Bayesian, ML and NJ), all methods yielded similar topologies and the same relationships for all major clades which were highly supported (Fig. 2A). According to network topology, the spectacled bear specimen clustered closer to *B. transfuga* and *B. schroederi*; while *B. procyonis* and *B. columnaris* conformed a separated subcluster within the genus *Baylisascaris*. These intra genus relatedness were confirmed by independent ITS1 (Figure 3B) and ITS2 (Figure 3A) rDNA analysis. In this later, partial sequences clustered *B. potosis* near to *B. procyonis* and *B. columnaris*; whereas sequences from *B. ailuri*, *B. transfuga* and *B. schroederi* were positioned as independent species nearest to sequences from *Baylisascaris* from the Venezuelan bear. Tree topologies were identical regardless the analytical approach (Bayesian, ML and Parsimony) and clades were highly supported. Interesting, the relationships of *Baylisascaris* species using ITS1 and ITS2 rDNA sequences displayed a closed association of the new *Baylisascaris* species from the spectacled bear *Tremarctos ornatus* with *B. transfuga* from the brown bear (*Ursus arctos*) and the polar bear (*Ursus maritimus*), *B. schroederi* from the giant panda (*Ailuropoda melanoleuca*) and *B. ailuri* from the red panda (*Ailurus fulgens*). These results display an interesting phylogenetic conservatism in the used of mammals host by this group of *Baylisascaris* species.

Nowadays, beside traditional features from

morphology, host and geographic origin, disease epidemiology and disease patterns and others, the description of new species or genotypes of parasites should considered molecular data of conserved and/or polymorphic DNA sequences from nuclear or mitochondrial origin.

Morphological characterization of our nematode showed that it belongs to *Baylisascaris* genus, worms first time recovered from wild spectacled bear, and clearly differing from *T. transfuga* (typical ascarid from bears) according with the number of pre-cloacal papillae. Molecular study of our nematodes from *T. ornatus* evaluated polymorphic sequences from the internal transcribed spacer (ITS1 and ITS2 rDNA), as well as sequences from the highly conserved 5.8S gene. Venezuelan specimen was clearly separated from other species of *Baylisascaris* in each of the analysis performed. Considering the endemicity of the host species, its restricted geographical location, with isolate populations exhibiting different genotypes depending on the geographical location (Ruíz-Martínez, 2003; Ruíz-Martínez *et al*., 2005), the origin of parasites (natural infection from a wild animal) and the morphological divergences at the caudal extreme of male, besides the remarkable results of the molecular genetic analysis and phylogenetic placement (with a new genotype clearly different from other *B. transfuga,* genotypes recorded), all together strongly suggest that this is a new *Baylisascaris* species for the South American spectacled bear *T. ornatus*, and we suggest the name of *Baylisascaris venezuelensis* considering this is the country in which the parasite was found. Beyond the parasitological finding, it becomes a new concern for venezuelan species-level conservation efforts for spectacled bear. Furthermore, it is known that *Baylisascaris procyonis* is the only well documented and most frequently cause of human and animal baylisascariosis. Even though there is no unequivocal evidence of naturally occurring of other *Baylisacaris* sp. in humans, all *Baylisascaris* species are potentially zoonotic. Concerns also raised for the more unknown potentially zoonotic *Baylisascaris* species, such as *B. transfuga* (Visser *et al.*, 2015) Thus, the zoonotic potential of *Baylisascaris*, and the existence of human invasion of the habitat of this endangered animal (even in National Park areas), in the venezuelan Andes represents a potential human health risk which must be taken in account.

Proposed new species:
Baylisascaris venezuelensis Pérez, García & Gauta, 2015
Host type: *Tremarctos ornatus*
Location: Bailadores (Mérida, Venezuela)
Voucher specimens: deposited on the Helminthological collection of the Center for Research in Veterinary Parasitology "Dr. Manuel Antonio Rivera Acevedo" (CIPV-MARA), School of Veterinary Sciences, Universidad Central de Venezuela, under the Catalogue number: CIPVMARA2010-N-EX-999.

Morphological features: Nematoda, Ascaridida.
Holotype: Male: stout nematodes, 10.2 cm length, posterior end slightly curled dorsally, tail round finished, with a little finger-like process; spicules: 0.9 mm, *area rugosa* present, 44 pre-cloacal papillae; anterior end: 3 lobes typical ascaroid-like with marked groves between lips and presence of dentigerous ridge, (triangle), cervical alae conspicuous, slightly curled and striated. Specimen divided into two fragments (anterior and posterior, lacking a two cm fragment in the middle half of the body (used in molecular analysis).
Allotype: Female: big, stout nematodes, whitish, pale yellow, 25 cm long. Head: 3 lobes with markedly surrounded dentigerous ridges, cervical alae conspicuous, slightly striated and curled.

Accession numbers for *Baylisascaris venezuelensis* sequences used in this article: (Kx151725 - Kx151727).

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