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RESEARCH ARTICLE

Morphological and molecular identification of the hard ticks parasitizing *Tremarctos ornatus* (Carnivora: Ursidae) from paramo of Ecuador

Sandra ENRÍQUEZ[1,2,7], María L. FÉLIX[3], Armando CASTELLANOS[4,5], Sergio BERMÚDEZ[6], José M. VENZAL[3]

[1]*Applied Entomology Unit, Zoonosis Research Institute, Central University of Ecuador, Quito, Ecuador*
[2]*Biodiversity, Zoonosis and Public Health Research Group (GIBCIZ), Central University of Ecuador, Quito, Ecuador*
[3]*Laboratorio de Vectores y Enfermedades Transmitidas, Departamento de Ciencias Biológicas, CENUR Litoral Norte, Universidad de la República, Salto, Uruguay*
[4]*Andean Bear Foundation, Quito, Ecuador*
[5]*Associate Researcher National Institute of Biodiversity (INABIO), Quito, Ecuador*
[6]*Department of Research in Medical Entomology, Gorgas Memorial Institute of Health Studies, Panama City, Panama*
[7]Corresponding author: ienriquez@uce.edu.ec

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ABSTRACT: The Andean bear or spectacled bear, *Tremarctos ornatus* (Cuvier), inhabits the Andes and is considered an endangered species due to anthropogenic factors. The aim of this study was to identify the tick species parasitizing the Andean bears in the evergreen shrubland and paramo grassland ecosystem in the Andes Mountain Range of Ecuador. Twenty-six ticks were removed from five Andean bears and morphologically identified as *Amblyomma multipunctum* Neumann, *Ixodes boliviensis* Neumann and *Ixodes montoyanus* Cooley. One specimen of each species was also molecularly analyzed and confirmed by BLAST. This study confirms the presence of *I. boliviensis* parasitizing *T. ornatus* and adds new records of *A. multipunctum* and *I. montoyanus* in its distribution. The parasite-host relationships are new in all cases for Ecuador.

Keywords: Ixodidae, *Amblyomma*, *Ixodes*, Andean bear, paramo, Ecuador.
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INTRODUCTION

Ticks are a group of Acari that parasite endothermic and ectothermic vertebrates, distributed in various tropical, subtropical and temperate environments (Guglielmone et al., 2020). In Ecuador, 34 species of hard ticks have been reported from the Galapagos Islands, the coastal region of the country, the Amazon Basin, and across an altitudinal gradient encompassing different types of forests and paramos in the Andes Mountain Range (Enríquez et al., 2020; Guglielmone et al., 2021; Paucar et al., 2022; Guglielmone et al., 2023). Other studies show the presence of ticks in anthropogenic and wilderness areas, as well as the pathogens detected in those ticks and the control measures (Labruna et al., 2013; Pesquera et al., 2015; Díaz et al., 2016; Rodriguez-Hidalgo et al., 2017; Gioia et al., 2018; Enríquez et al., 2020; Maya-Delgado et al., 2020; Aguilar-Domínguez et al., 2021; Paucar et al., 2022; Pilatasig, 2022).

The taxonomy and ecology of Ecuadorian ticks remain poorly understood, especially of those species inhabiting remote areas and parasitizing wild vertebrates. One of these wild vertebrates is the Andean or spectacled bear, *Tremarctos ornatus* (Cuvier), which inhabits the Andes, from Venezuela to northern Argentina, at elevations ranging from 800 to over 4500 meters (Sandoval-Guillén and Yánez-Moretta, 2019). In Ecuador, the distribution of this bear occurs in evergreen shrubland and paramo grassland ecosystem (paramo) (Galeas et al., 2013), temperate and subtropical forests between 1000 and 4300 meters, and prefers to inhabit cloud forests (Castellanos, 2010). According to the International Union for Conservation of Nature (IUCN, 2022), the conservation status of *T. ornatus* is Vulnerable (VU) at the global level, but in Ecuador, it is considered as an endangered species (EN) due to the loss and fragmentation of its habitats, the expansion of the agricultural frontier, mining, forest fires and hunting (Tirira, 2017).

The interaction between ticks and Andean bears is poorly understood throughout their entire distribution area and until now only *Ixodes boliviensis* Neumann was reported on this bear in Colombia (Anonymous, 1998; Guglielmone et al., 2021). Recently Carvajal and Castellanos (2021) reported *Amblyomma mixtum* Koch (1 female and 9 "female nymphs") and *Ixodes fuscipes* Koch (13 females) in *T. ornatus* from Ecuador.

The aim of this study was to identify morphologically and molecularly the tick species parasitizing *T. ornatus* of Ecuador and to re-evaluate the identification of ticks reported by Carvajal and Castellanos (2021).

MATERIALS AND METHODS

Ticks collections and morphological re-identification

Ticks identified in this study were removed from Andean bears following the protocol used by Castellanos et al. (2018). Bears examined and released were part of the Research Projects: "Systematics, Ecology, Reproductive Biology and Genomics of the Mammals of Ecuador" and

Table 1. Descriptions of localities of Ecuador.

Province	Locality	Altitude	Number of bears
Pichincha	Papallacta, Cayambe-Coca National Park (CCNP)	3600 m	1
Napo	Cosanga, Antisana National Park (ANP)	2000 m	1
Tungurahua	Patate, Valle Manteles-Leito, Llanganates National Park (LNP)	3190 m	3

"National Mammal Collection Program", which were approved through via Collection Permits: No. MAE-DNB-CM-2019-0126, No. MAAE-ARSFC-2020-0642, and No. MAAE-ARSFC-2021-1644 (Ministry of Environment and Water of Ecuador). The description of the sample localities is shown in Table 1.

All ticks identified by Carvajal and Castellanos (2021) (24 individuals) were again reviewed by one of us (SE) plus two newly collected ticks. Ticks were identified morphologically with a stereo microscope (NIKON model SMZ745T, Tokyo, Japan) (magnifications ×0.67–5), using the taxonomic keys for adult *Amblyomma* (Onofrio et al., 2006a) and *Ixodes* (Onofrio et al., 2006b), and the original descriptions of *Ixodes montoyanus* Cooley (Cooley, 1944; Keirans, 1973) and *Amblyomma multipunctum* Neumann nymphs (Labruna et al., 2013). Tick photographs were taken by a stereomicroscope (NIKON model SMZ745T, Tokyo, Japan) with a camera (Mshot model MS60, Guangzhou, China) and edited with Adobe Photoshop CC 2019.

Molecular identification

Due to the morphological differences found during the re-identification of the material collected from *T. ornatus*, 11 ticks were identified molecularly (nine from the Carvajal and Castellanos collections and two new specimens collected). Individual ticks were bisected longitudinally using sterile scalpels and washed with distilled water to remove ethanol. DNA was extracted using the commercial kit GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) following manufacturer instructions. To compare with other Neotropical ticks, the specimens were analyzed through PCR amplification of a ~460 base pair (bp) fragment of the tick mitochondrial *16S rRNA* gene (Black and Piesman, 1994). Amplicons of the expected size of two *Ixodes* females and one *Amblyomma* nymph identified by Carvajal and Castellanos (2021) were purified, and Sanger sequenced (Macrogen, South Korea). Obtained sequences were edited using MEGA 7 (Kumar et al., 2016), and comparisons with GenBank available sequences were made by the basic local alignment search tool (BLAST) (Altschul et al., 1990).

RESULTS

Morphological and molecular identification

Twenty-six ticks were morphologically identified, corresponding to *A. multipunctum* (11 nymphs), *I. boliviensis* (5 females and 1 male), and *I. montoyanus* (9 females); of these, all ticks identified by Carvajal and Castellanos (2021) were re-evaluated plus two newly collected ticks (Table 2). The principal morphological characteristics to identify *A. multipunctum* nymphs were described by Labruna et al. (2013). Moreover, some characteristics of *I. montoyanus* females were compiled with the works of Cooley (1944), Onofrio et al. (2006b), Apanaskevich and Bermúdez (2017) and Onofrio et al. (2020).

Females of *I. boliviensis* were recognized when compared with the characteristics presented by Onofrio et al. (2006b) because is the more precise work to identify *Ixodes* specimens of South America. In the case of the male, we present here a preliminary description of the unique exemplar collected on one Andean bear in this study. In this sense, our specimen was 3.0 mm long (from the apical part of the hypostome to posterior body margin), a maximum breadth of 1.5 mm; oval idiosoma; dorsal basis of capitulum without cornua, hypostome short, broad, and apically rounded with dentition 4/4 arranged in transversal rows; auriculae small and sharp; scutum with deep punctuations distributed at the margins and in the posterior region; coxa I with two spurs, the internal spur long, extending beyond the middle of coxa II and the external spur triangular small; coxa II with two triangular small spurs; coxae III-IV with a small triangular external spur; genital aperture situated at the level of the third coxa; median plate with deep punctuations evenly distributed, adanal and anal plates with fine punctuations; spiracular plate broadly oval; a ventral region with numerous long white setae (Figs 1-3).

The sequence obtained of one *Amblyomma* nymph (Al-AmuN30) showed 98.30% (404/411 bp) and 98.29% (402/409) identity with *A. multipunctum* from Ecuador (GenBank KC677673 and KJ584366). Regarding *Ixodes* ticks, one sequence (Co-IboH33) showed 99.03% (408/412 bp) identity with *I. boliviensis* (GenBank KM077437), and the sequence obtained from the female of *I. montoyanus* (Al-ImH28) showed 100% (409/409 bp) identity with *Ixodes* sp. AMP-2014 isolate 8C1, both from sequenced reports of Ecuador (GenBank KM077438). Sequences from the *16S rDNA* gene for this study are available in GenBank for *A. multipunctum* (accession number OQ557626); *I. boliviensis* (accession number OQ557627) and *I. montoyanus* (accession number OQ557628). These whole or partially dissected specimens were deposited in the National Reference Collection of Arthropods of Importance in Zoonoses (CONRAZ) of the Zoonosis Research Institute (CIZ), Central University of Ecuador, Quito.

DISCUSSION

Due to the analysis of several factors such as the difficulty of identifying sex in immature stages (larvae and nymphs) of hard ticks (Polanco-Echeverry and Ríos-Osorio, 2016), the presence of an ornate scutum in females of the species *A. mixtum*, its altitudinal distribution (Nava et al., 2014),

Table 2. Morphological and molecular identification of ticks parasitizing Andean bears from Ecuador.

Locality	Sample ID ˣ	Ticks stage	Morphological ID	Molecular ID (GenBank Accession)
CCNP	Da-AmuN01	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN02	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN03	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN04	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN05	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN06	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN07	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN08	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN09	Nymph*	*Amblyomma multipunctum*	
CCNP	Da-AmuN10	Nymph*	*Amblyomma multipunctum*	
ANP	Co-IboH31	Female*	*Ixodes boliviensis*	
ANP	Co-IboH32	Female*	*Ixodes boliviensis*	
ANP	Co-IboH33	Female*	*Ixodes boliviensis*	*Ixodes boliviensis* (OQ557627)
ANP	Co-IboH34	Female*	*Ixodes boliviensis*	
LNP	Al-ImH23	Female*	*Ixodes montoyanus*	
LNP	Al-ImH24	Female*	*Ixodes montoyanus*	
LNP	Al-ImH25	Female*	*Ixodes montoyanus*	
LNP	Al-ImH26	Female*	*Ixodes montoyanus*	
LNP	Al-ImH27	Female*	*Ixodes montoyanus*	
LNP	Al-ImH28	Female*	*Ixodes montoyanus*	*Ixodes montoyanus* (OQ557628)
LNP	Al-ImH29	Female*	*Ixodes montoyanus*	
LNP	Al-AmuN30	Nymph*	*Amblyomma multipunctum*	*Amblyomma multipunctum* (OQ557626)
LNP	De-ImH01	Female*	*Ixodes montoyanus*	
LNP	De-ImH02	Female*	*Ixodes montoyanus*	
LNP	P4-IbolH35	Female⁺	*Ixodes boliviensis*	
LNP	P4-IbolM36	Male⁺	*Ixodes boliviensis*	

ˣEach letter represents a captured Andean bear individual, *Samples identified by Carvajal and Castellanos (2021) and re-identified by the authors, ⁺Samples identified only by the authors.

and its type of hosts (Guglielmone et al., 2021), arose doubts about the identification of Carvajal and Castellanos (2021). For these reasons, it was necessary to review again the identification of these ticks, including the use of molecular analysis.

During this study, the presence of *I. boliviensis* parasitizing *T. ornatus* is confirmed and new records of *A. multipunctum* and *I. montoyanus* are added to its distribution, this increments the parasite-host relationships, which are new in all cases for Ecuador. Consequently, the molecular identifications of *A. multipunctum* nymphs, *I. boliviensis*, and *I. montoyanus* adults, replace the identifications of *A. mixtum* and *I. fuscipes* reported by Carvajal and Castellanos (2021).

It is essential to highlight that *A. mixtum* is an eclectic species with a wide distribution from the southern United States (Texas) to west-northern South America and some Caribbean Islands (Guglielmone et al., 2021). In Ecuador, this species is known from western Ecuador (provinces of El Oro, Guayas, Los Ríos, Manabí and Pichincha, see Nava et al., 2014 and Paucar et al., 2022), in environments such as dry, semiarid, and riparian forests, or savanna lowlands instead of Andean cloud forests or paramo environments (Estrada-Peña et al., 2014; Aguilar-Domínguez et al., 2021). Regarding *I. fuscipes*, its distribution is restricted to three southern Brazilian states (Paraná, Rio Grande do Sul and São Paulo) and Uruguay (Cerro Largo, Florida, Lavalleja, Maldonado, Rocha and Tacuarembó) (Guglielmone et al., 2021). This tick has been widely confused with *Ixodes spinosus* Neumann, while and *Ixodes aragaoi* Fonseca is a synonym of *I. fuscipes* (Guglielmone et al., 2020; Labruna et al., 2020).

On the other hand, *A. multipunctum* occurs in highlands, humid montane forest areas, associated with the distribution of its primary host the mountain tapir, *Tapirus pinchaque* (Roulin) (Labruna et al., 2013; Pesquera et al., 2015; Guglielmone et al., 2021). This tapir species inhabits montane forests and paramos (from 1500 to 4800 m) of Colombia, Ecuador and Peru (Castellanos-Peñafiel et al., 2021) sharing habitat with the spectacled bear; for this reason, *A. multipunctum* might have parasitized both host species, particularly by the immature stages of this tick. The nymph analyzed here, showed more than 98% of identity with the DNA sequences of adults from Ecuador. This is the first host record for *A. multipunctum* nymphs on Andean bears. Regarding *I. boliviensis*, molecular differences between Central America and Southern America populations, indicate the presence of at least two different species (Bermúdez et al., 2021).

Figures 1-3. *Ixodes boliviensis* (male)—**1.** Dorsal view, **2.** Ventral view, **3.** Ventral view of capitulum and idiosoma.

In the present study, there is a preliminary sketch of males and females from Ecuador, which also show some morphological differences regarding the size and dental formula, but without genetic differences. Previously in Ecuador, Pesquera et al. (2015) found one female of *I. boliviensis* on cattle in Papallacta (paramo) at 3300 m of altitude, one of the areas where the exemplars of this study were collected. In addition, Paucar et al. (2022) found females of *I. boliviensis* parasitizing cattle in three farms of Quijos River Valley (Napo province) and Northwest Pichincha province. These areas are in the east and west foothills of the Andes Mountains, respectively at ranging altitudes from 800 to 1600 m. Thus, this geographical and biological information suggests the identification of *I. boliviensis* in Ecuador, although new research is still necessary to define the current status of this species. Also, it will be necessary to carry out exhaustive studies for the formal description of the male of this species.

We report for the first time the presence of *I. montoyanus* on two Andean bears of Ecuadorian paramo. Previously this tick was reported from cattle in the same areas where *I. boliviensis* was found (Paucar et al., 2022). This information contributes to extending the list of hosts of *I. montoyanus* adults because until now just mammals of families Cervidae and Procyonidae were considered as hosts (Guglielmone et al., 2021). It should be noted that a morphological characteristic to identify specimens of *I. montoyanus* is the presence of membranous areas (syncoxa) and the absence of external spur in the coxae I-II. The syncoxae was not mentioned in the original description by Cooley (1944) and Keirans (1973) but is notified by Apanaskevich and Bermúdez (2017) and Onofrio et al. (2020). Due to these structures, females of *I. montoyanus* resemble *Ixodes tapirus* Kohls, *Ixodes lasallei* Méndez & Ortiz, *Ixodes venezuelensis* Kohls, *Ixodes guatemalensis* Kohls, *Ixodes bocatorensis* Apanaskevich & Bermúdez, and *Ixodes catarinensis* Onofrio & Labruna but differs from those species by the long and hooked auriculae, scutal punctuations, size, and form of the porose areas (Apanaskevich et al., 2017; Onofrio et al., 2020). The DNA sequence of *I. montoyanus* is the first sequence verified for this species. With this morphological, biological, and molecular information, we confirm the presence of this tick on the Andean bear from the paramo of Ecuador. Finally, in addition to an adequate taxonomic and morphological review of ticks, many aspects of their distribution and ecology should be assessed.

Authors' contributions

Sandra Enríquez: Conceptualization, morphological identification of the ticks, writing. **María L. Félix:** Molecular analysis, investigation; validation. **Armando Castellanos:** Funding acquisition for fieldwork, field collection of the ticks. **Sergio Bermúdez:** Visualization, writing-original draft, methodology. **José M. Venzal:** Methodology, data curation, resources. All authors participate in the final revision and writing of the manuscript.

Statement of ethics approval

The present study was conducted according to the legal requirements of Ecuador. Ministry of Environment and Water of Ecuador via Collection Permits No. MAE-DNB-CM-2019-0126, No. MAAE-ARSFC-2020-0642 and No. MAAE-ARSFC-2021-1644 authorized ticks capture on Andean bears. All animals were treated carefully and then released into their habitats.

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Conflict of interest

The authors declare that there is no any conflict of interest.

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