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Molecular characterization of novel *Ehrlichia* genotypes in *Ixodes auritulus* from Uruguay

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ABSTRACT

Ehrlichia are small intracellular Gram-negative bacteria transmitted by ticks. These microorganisms cause ehrlichiosis, a complex of life-threatening emerging zoonoses and diseases of global veterinary relevance. The aim of this study was to investigate the presence of *Ehrlichia* in free-living *Ixodes auritulus* collected in Uruguay. Ticks were collected from vegetation in five localities from the southeast and northeast of the country between 2014 and 2017. Detection of *Ehrlichia* DNA was performed in pools of adults or nymphs grouped according to the collection site and date. A total of 1,548 *I. auritulus* ticks were collected in four of the five locations sampled. Fragments of three loci (*16S* rRNA, *dsb* and *groEL*) were obtained by PCR, and phylogenies inferred using Bayesian inference analysis for each gene independently. DNA of *Ehrlichia* spp. was found in 15 out of 42 tick pools. Based on the topology of the phylogenetic trees, our sequences represent two novel genotypes for the genus named as *Ehrlichia* sp. Serrana and *Ehrlichia* sp. Laguna Negra. Both genotypes were closely related to *Ehrlichia* sp. Magellanica, a species detected in *Ixodes uriae* and Magellanic penguins. Considering that all stages of *I. auritulus* and *I. uriae* are parasites of birds, their phylogenetic relationships, and common eco–epidemiological profiles, it is reasonable to state that these genotypes of *Ehrlichia* spp. may represent a natural group likely associated with birds. Our results constitute the first characterization of *Ehrlichia* spp. in Uruguay. Future studies on birds reported as hosts for *I. auritulus* are needed to further understand the epidemiological cycles of both *Ehrlichia* genotypes in the country. Finally, *I. auritulus* does not feed on humans, so the two *Ehrlichia* species reported herein might have no implications in human health.

1. Introduction

The order Rickettsiales (Alphaproteobacteria) includes obligate intracellular parasites that infect a variety of invertebrate and vertebrate hosts. Rickettsiales comprises the families *Anaplasmataceae*, *Midichloriaceae* and *Rickettsiaceae* (Montagna et al., 2013; Szokoli et al., 2016). The family *Anaplasmataceae* is currently divided into five established and two *Candidatus* genera: *Anaplasma*, *Aegyptianella*, *Ehrlichia*, *Neorickettsia*, *Wolbachia*, "*Candidatus* Neoehrlichia", and "*Candidatus* Xenohaliotis" (Thomas et al., 2016).

Ehrlichia are small Gram-negative tick-transmitted bacteria that form microcolonies within membrane-bound cytoplasmic vacuoles called morulae (Popov et al., 1998). These bacteria are the agents of ehrlichiosis, a complex of life-threatening emerging zoonoses and diseases of veterinary importance worldwide (Esemu et al., 2011). *Ehrlichia* species differ in their target cells both in mammals (monocytes, neutrophils or endothelial cells) and ticks (salivary glands, intestinal epithelium, and hemolymph) (Brouqui & Matsumoto, 2007; Aguiar, 2017). In contrast to *Rickettsia* spp., there is no evidence of transovarial transmission in *Ehrlichia* spp. in ticks (Ismail & McBride, 2017). Instead,

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ticks become infected with *Ehrlichia* spp. while feeding on infected vertebrate reservoirs and the infection is perpetuated transstadially (i.e. larva-nymph-adult) (Ismail & McBride, 2017). Human and animal ehrlichiosis are caused worldwide by six *Ehrlichia* species, namely *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ewingii*, *Ehrlichia ruminantium*, and *Ehrlichia minasensis* (see Aguiar, 2017). In the Southern Cone of South America, *E. canis*, the etiological agent of canine monocytic ehrlichiosis, is the most commonly reported species of this genus (López et al., 2012; Lasta et al., 2013; Cicuttin et al., 2015).

Additionally, different strains closely related to *E. chaffeensis* were detected in *Amblyomma parvum*, *Amblyomma tigrinum* and in marsh deer (*Blastocerus dichotomus*) in Argentina (Tomassone et al., 2008; Cicuttin et al., 2017; Guillemi et al., 2019; Monje et al., 2019). Moreover, *Ehrlichia* sp. strain Cordoba was detected in *A. tigrinum* ticks (Cicuttin et al., 2017). Recently, also in Argentina, *A. tigrinum*, *Amblyomma triste* and *Amblyomma neumanni* were found positive after molecular screenings targeting this bacterium, and three novel strains (*Ehrlichia* sp. strain Iberá, *Ehrlichia* sp. strain Delta, and *Ehrlichia* sp. strain La Dormida) were identified (Cicuttin et al., 2020; Eberhardt et al., 2020; Fargnoli et al., 2020).

In Brazil, reports include the detection of *Ehrlichia* cf. *chaffeensis* in *B. dichotomus* (Sacchi et al., 2012), and some *Ehrlichia* spp. strains pending further molecular characterization were detected in wild carnivores (Widmer et al., 2011; Almeida et al., 2013) and a horse (Vieira et al., 2016). Cattle and *Rhipicephalus microplus* ticks maintain *E. minasensis* infections in Brazil (Cabezas-Cruz et al., 2016; Aguiar, 2017) and recently, *Ehrlichia* sp. was detected in *Amblyomma sculptum* in the Brazilian Pantanal (Muraro et al., 2021).

In Chile, a novel genotype, *Ehrlichia* sp. Magellanica, seems to represent a bird-associated lineage within the genus, since it was

Table 2
Data for *Ixodes auritulus* collected and detection of *Ehrlichia* spp.

Collection site	Date	Stage	No. of ticks	Pools	Positive pools	Positive pools code	*Ehrlichia* genotype	GenBank accession numbers		
								16S rRNA	*dsb*	*groEL*
Gruta de los Cuervos (T)	August 2016	Nymph	1	1	0					
	December 2016	Female	1	1	0					
	July 2017	Nymph	1	1	0					
Amarillo (Ri)	October 2016	Female	1	1	1	S12IaH4[a]	Serrana	MW628647[a]	MW650903[a]	
		Nymph	1	1	0					
	June 2017	Female	1	1	1	S17IaH1[a]	Serrana	MW628649[a]	MW650901[a]	MW650909[a]
		Nymph	16	3	0					
		Larva	6	#	#					
Lunarejo (Ri)	October 2016	F-N-L	0	0	0					
	December 2016	F-N-L	0	0	0					
	April 2017	F-N-L	0	0	0					
	July 2017	F-N-L	0	0	0					
Reserva Natural Salus (La)	May 2016	Nymph	48	5	1	S10IaN9[a]	Serrana		MW650902[a]	MW650910[a]
	December 2016	Female	6	1	0					
		Nymph	5	1	0					
	July 2017	Nymph	12	2	1	S17IaN33[a]	Serrana	MW628650[a]	MW650904[a]	
		Larva	440	#	#					
Laguna Negra (Ro)	March 2014	Female	4	1	1	S5IaH4	Serrana			
		Nymph	9	1	0					
		Larva	4	#	#					
	May 2014	Nymph	30	3	2	S6IaN9; S6IaN11	Serrana (2)			
		Larva	144	#	#					
	November 2015	Female	4	1	1	S6IaH12[a]	Laguna Negra	MW628646[a]	MW650906[a]	MW650908[a]
	August 2014	Female	6	1	0					
		Nymph	47	5	1	S7IaN19	Serrana			
		Larva	665	#	#					
	November 2014	Female	50	10	5	S8IaH27[a]; S8IaH28; S8IaH31; S8IaH33; S8IaH34	Laguna Negra (1); Serrana (4)			MW650907[a]
		Nymph	16	2	1	S8IaN38[a]	Serrana	MW628648[a]	MW650905[a]	
		Larva	30	#	#					
Total			1548	42	15					

Abbreviations: T, Tacuarembó; Ri, Rivera; La, Lavalleja; Ro, Rocha; #, larvae are not included in pools; F, Female; N, Nymph; L, Larva.
[a] Sequences of positive pools used for phylogenetic tree construction.

Table 1
PCR primers used to amplify the partial *16S* rRNA, *groEL*, and *dsb* genes of *Ehrlichia* spp.

Primer name	Targeted gene	Sequence	Amplicon size (bp)	Reference
EHR16SD[a]	*16S* rRNA	GGTACCYACAGAAGAAGTCC	345	Parola et al. (2000)
EHR16SR[a]		TAGCACTCATCGTTTACAGC		Parola et al. (2000)
fD1		AGAGTTTGATCCTGGCTCAG	~1,500	Weisburg et al. (1991)
Rp2		ACGGCTACCTTGTTACGACTT		Weisburg et al. (1991)
HS1a	*groEL*	AITGGGCTGGTAITGAAAT	~1,400	Sumner et al. (1997); Nicholson et al. (1999)
HS6a		CCICCIGGIACIAIACCTTC		Sumner et al. (1997); Nicholson et al. (1999)
HS43		ATWGCWAARGAAGCATAGTC	1,297	Lotric-Furlan et al. (1998)
HSVR		CTCAACAGCAGCTCTAGTAGC		Lotric-Furlan et al. (1998)
Dsb-330	*dsb*	GATGATGTTTGAAGATATSAAACAAAT	401	Doyle et al. (2005); Almeida et al. (2013)
Dsb-720[b]		CTATTTTACTTCTTAAAGTTGATAWATC	349	Doyle et al. (2005); Almeida et al. (2013)
Dsb-380		ATTTTTAGRGATTTTCCAATACTTGG		Doyle et al. (2005); Almeida et al. (2013)

[a] Primers used in the initial PCR screening.
[b] Primer used in the first and second round.

detected in *Ixodes uriae* collected on Magellanic penguins (*Spheniscus magellanicus*) (Muñoz-Leal et al., 2019).

While the occurrence of ehrlichiae in Uruguay is uncertain, Conti-Díaz (2001) referred to possible cases of ehrlichiosis in humans. Although the disease is listed as emergent in the country, infection in animals and vectors has not been investigated.

Ixodes auritulus is a cosmopolitan tick species distributed through the Afrotropical, Australasian, Nearctic, and Neotropical Zoogeographic Regions and all stages parasitize mainly birds (Nava et al., 2017; Guglielmone et al., 2020). The presence of *Borrelia burgdorferi* (*sensu lato*) was recently reported in *I. auritulus* from Argentina and Uruguay (Cicuttin et al., 2019; Carvalho et al., 2020); however, information about the presence of other pathogens is scarce.

As *Ixodes* spp. also can transtadially maintain bacteria of the family *Anaplasmataceae*, the aim of this study was to investigate the presence of *Ehrlichia* in free-living *I. auritulus* ticks collected in Uruguay.

2. Materials and methods

2.1. Study area

Fieldwork was conducted at five localities in Uruguay between 2014 and 2017. Three localities, Gruta de los Cuervos (−31.618888, −56.046389), Tacuarembó Department; Amarillo (−31.663611, −55.050555) and Lunarejo (−31.141388, −55.900277), Rivera Department, are located in the northeast region of the country, within the

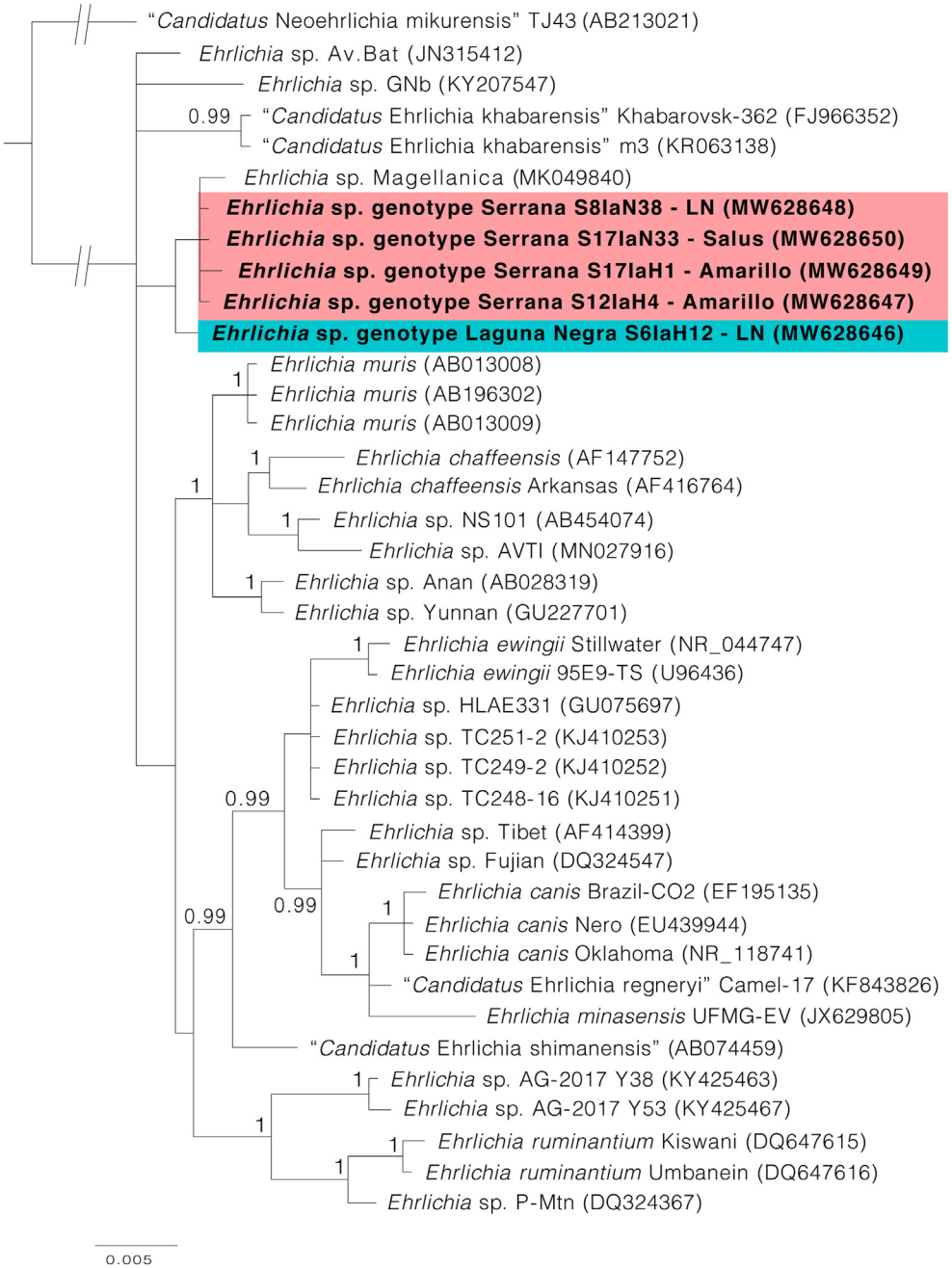

Fig. 1. Bayesian phylogenetic analysis inferred for partial fragments of the *16S* rRNA gene. Bayesian posterior probabilities > 0.95 are indicated above or below the nodes. The positions of *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra are highlighted within red and blue boxes, respectively. The scale-bar indicates the number of substitutions per nucleotide position. GenBank accession numbers are in parentheses

Uruguayan ecoregion Gondwanic Sedimentary Basin (Brazeiro et al., 2012). The other two localities, Reserva Natural Salus (−34.421111, −55.315000), Lavalleja Department, and Laguna Negra (−34.085833, −53.738055), Rocha Department, are in the southeast part of the country, and belong to the ecoregion Sierras del Este *sensu* Brazeiro et al., (2012).

2.2. Tick collection and identification

Questing ticks were collected from vegetation using the flagging method. The collection was carried out for a period of 2 h in the samplings in each visit to the sites. Ticks were picked up from the cloth at 5–10-m intervals and stored in plastic tubes with 95% ethanol. In the laboratory, each arthropod was identified using a stereomicroscope following the morphological keys for larval, nymph and adult stages by Kohls (1960), Webb et al. (1990), and Nava et al. (2017).

2.3. DNA extraction and detection of Ehrlichia spp.

Only free-living nymphs and adult ticks were used in this study. Since *Ehrlichia* spp. are not maintained by transovarial transmission, larvae were not analyzed.

Detection of *Ehrlichia* DNA was performed into pools of adult or nymphs (1–10 ticks per pool) separated according to site and collection date. Briefly, ticks were rinsed with distilled water to remove ethanol and bisected longitudinally using sterile scalpel blades and forceps. Finally, each pool was homogenized cutting thoroughly the ticks with dissecting scissors. DNA was extracted using the commercial kit GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) following the manufacturer's instructions. The concentration and purity of DNA was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular screening was carried out using primers EHR16SD/EHR16SR with PCR conditions described by Parola et al. (2000). This PCR enables detection of a *16S* rRNA gene fragment of members of the family *Anaplasmataceae* including the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*. Positive samples were subjected to four additional PCR protocols. Two overlapping fragments were obtained using primers fD1/EHR16SR and EHR16SD/Rp2 (Weisburg et al., 1991; Inokuma et al., 2001) to amplify nearly full-length sequence of the *16S* rRNA gene. In addition, a semi-nested and nested PCRs targeting *dsb* (disulfide oxidoreductase) and *groEL* (60 kDa chaperonin) genes, respectively, were performed (Sumner et al., 1997; Lotric-Furlan et al., 1998; Nicholson et al., 1999; Doyle et al., 2005; Almeida et al., 2013). All primers used and fragment sizes are listed inTable 1. Distilled water and an *Ehrlichia canis* DNA-positive sample were included as negative and positive controls in all runs. Five microliters of PCR products were analyzed by electrophoresis into 1.5% agarose gels, stained with GoodView™ Nucleic Acid Stain (Beijing SBS Genetech Co., Ltd.), and examined under UV transillumination. Amplicons were purified using GeneJET PCR purification kit (Thermo Scientific, Lithuania) and sent for sequencing to Macrogen (Seoul, Korea). BLASTn analyses (www.ncbi.nlm.nih.gov/blast) were

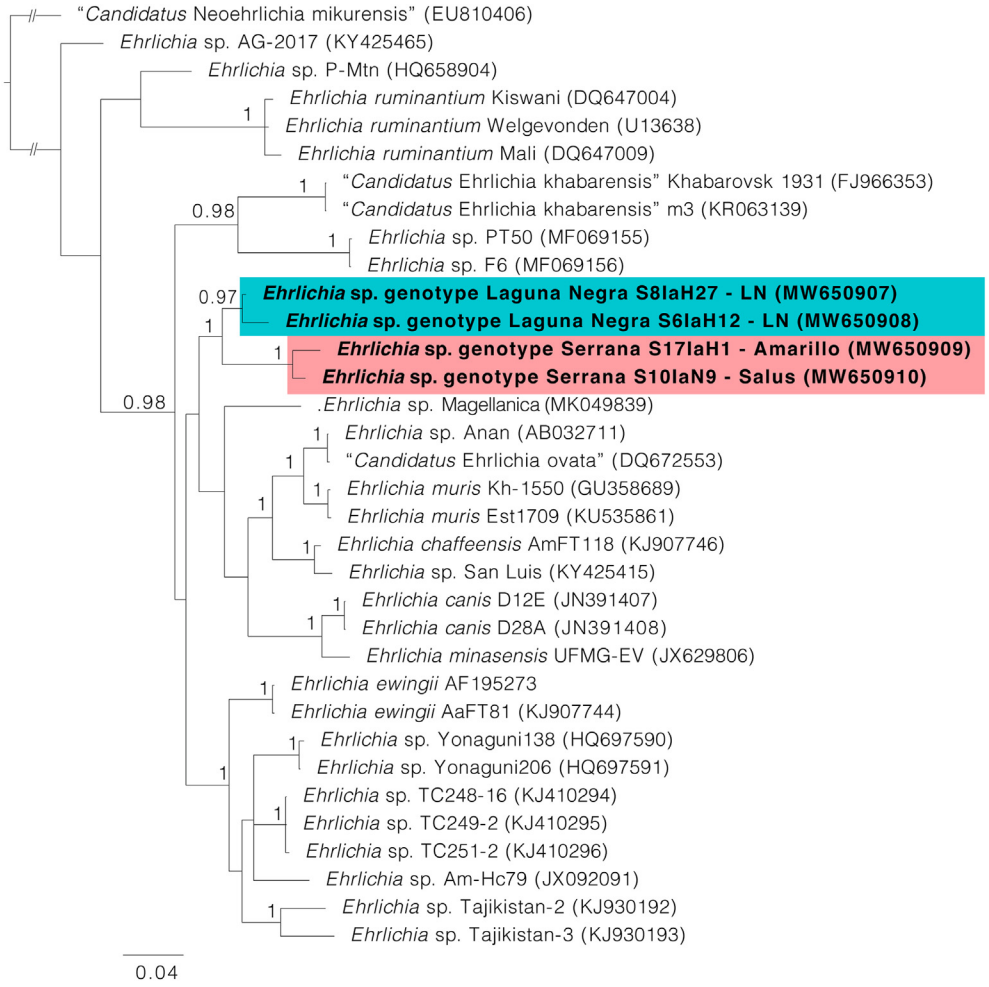

Fig. 2. Bayesian phylogenetic analysis inferred for partial fragments of the *groEL* gene. Bayesian posterior probabilities > 0.95 are indicated above or below the nodes. The positions of *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra are highlighted within red and blue boxes, respectively. The scale-bar indicates the number of substitutions per nucleotide position. GenBank accession numbers are in parentheses

performed in order to infer closest identities with microorganisms available on GenBank database (Altschul et al., 1990), and to include these sequences into a phylogenetic analysis. Nucleotide identities of obtained sequences were calculated using the Sequence Identity and Similarity (SIAS) calculator (http://imed.med.ucm.es/Tools/sias.html).

2.4. Phylogenetic analyses

Independent alignments using obtained sequences for *16S* rRNA, *dsb* and *groEL* loci were constructed with CLUSTAL W (Thompson et al., 1994) including homologue sequences downloaded from GenBank. Three phylogenetic trees were inferred using Bayesian inference analysis as implemented in MrBayes 3.2.5. (Huelsenbeck & Ronquist, 2001), using 1,000,000 generations. The general time reversible (GTR) model was chosen to run all the trees. Each tree was sampled every 100 generations, begun with random seeds and ran four times. The first 25% of the trees was discarded as "burn-in", and the remaining subset of trees was used to calculate Bayesian posterior probabilities. Sequences of "*Candidatus* Neoehrlichia mikurensis" (EU810406; AB213021) and *Ehrlichia ruminantium* (AF308669) were used to root the phylogenetic trees.

3. Results

3.1. Tick collection

A total of 1,548 *I. auritulus* ticks (73 females, 185 nymphs and 1,290 larvae) were collected on vegetation at four of the five locations sampled (Table 2). Other tick species such as *Amblyomma aureolatum*, *Haemaphysalis juxtakochi*, and *Ixodes fuscipes* were also collected but not included in this study.

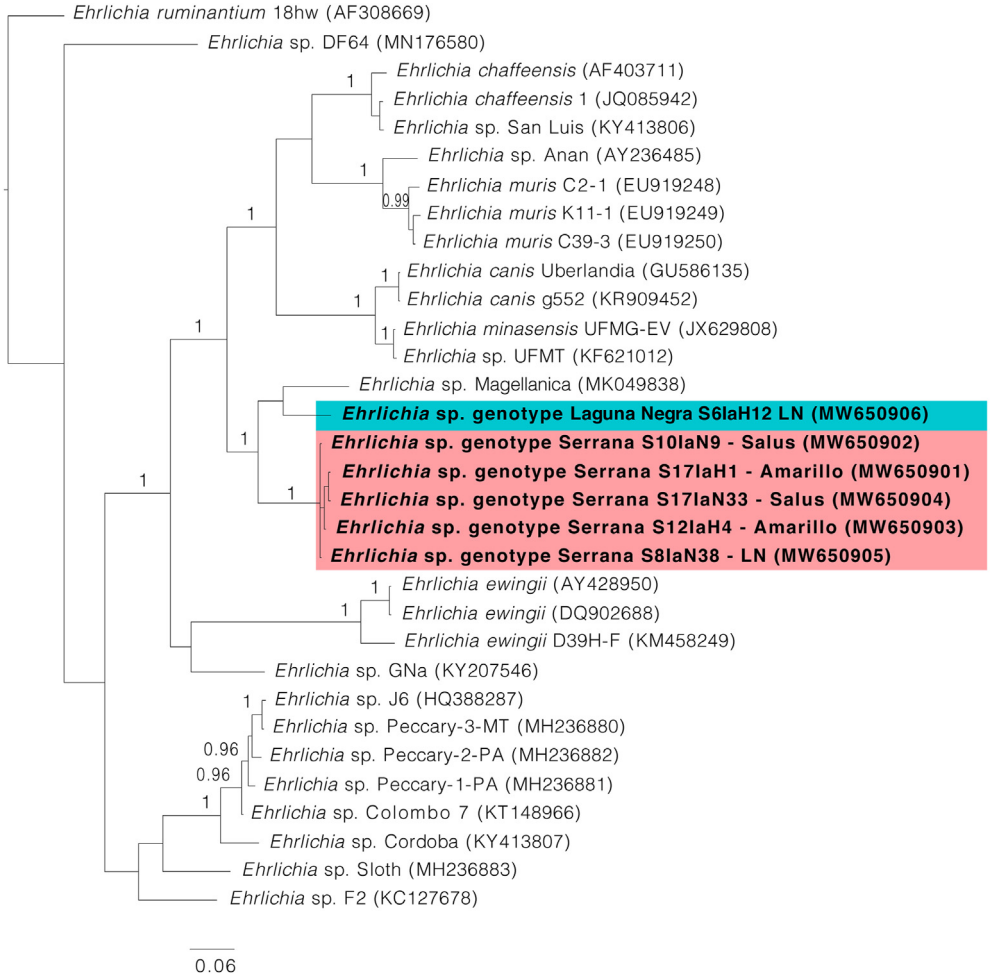

Fig. 3. Bayesian phylogenetic analysis inferred for partial fragments of the *dsb* gene. Bayesian posterior probabilities > 0.95 are indicated above or below the nodes. The positions of *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra are highlighted within red and blue boxes, respectively. The scale-bar indicates the number of substitutions per nucleotide position. GenBank accession numbers are in parentheses

Table 3
Pairwise comparison matrix for *16S* rDNA sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica

		1	2	3	4	5	6
1	*Ehrlichia* sp. Laguna Negra S6IaH12 (LN)	–					
2	*Ehrlichia* sp. Serrana S8IaN38 (LN)	99.79	–				
3	*Ehrlichia* sp. Serrana S12IaH4 (Amaril)	99.78	100	–			
4	*Ehrlichia* sp. Serrana S17IaH1 (Amarillo)	99.68	99.89	99.89	–		
5	*Ehrlichia* sp. Serrana S17IaN33 (Salus)	99.78	100	100	99.89	–	
6	*Ehrlichia* sp. Magellanica (MK049840)	97.12	97.12	90.8	92.24	92.52	–

Table 4
Pairwise comparison matrix for *dsb* sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica

		1	2	3	4	5	6	7
1	*Ehrlichia* sp. Laguna Negra S6IaH12 (LN)	–						
2	*Ehrlichia* sp. Serrana S8IaN38 (LN)	88.47	–					
3	*Ehrlichia* sp. Serrana S10IaN9 (Salus)	88.47	100	–				
4	*Ehrlichia* sp. Serrana S12IaH4 (Amarillo)	88.47	100	100	–			
5	*Ehrlichia* sp. Serrana S17IaH1 (Amarillo)	88.16	99.68	99.68	99.68	–		
6	*Ehrlichia* sp. Serrana S17IaN33 (Salus)	88.16	99.68	99.68	99.68	100	–	
7	*Ehrlichia* sp. Magellanica (MK049838)	88.78	87.22	87.22	87.22	86.91	86.91	–

Table 5
Pairwise comparison matrix for *groEL* sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica

		1	2	3	4	5
1	*Ehrlichia* sp. Laguna Negra S6IaH12 (LN)	–				
2	*Ehrlichia* sp. Laguna Negra S8IaH27 (LN)	99.78	–			
3	*Ehrlichia* sp. Serrana S10IaN9 (Salus)	96.05	95.83	–		
4	*Ehrlichia* sp. Serrana S17IaH1 (Amarillo)	95.61	95.39	98.02	–	
5	*Ehrlichia* sp. Magellanica (MK049839)	92.85	92.51	92.17	91.83	–

3.2. Detection of Ehrlichia DNA in ticks

Ticks were processed and analyzed in 42 pools (17 containing females and 25 containing nymphs). Of these, 15 (9 containing females and 6 containing nymphs) were positive for DNA of *Ehrlichia* spp. Geographically, *Ehrlichia* spp. DNA was detected in Amarillo (two pools of females), Reserva Natural Salus (two pools of nymphs) and Laguna Negra (seven pools of females and two of nymphs) (Table 2).

3.3. Phylogenetic analyses and nucleotide comparisons of sequences

Trees inferred by Bayesian analysis were constructed to define the phylogenetic position of the generated sequences. With the exception of the *groEL* tree, our sequences clustered into a monophyletic group including *Ehrlichia* sp. Magellanica from Chile (Figs. 1–3). However, sequences for all three loci were obtained from only two pools (S6laH12 and S17laH1) (Table 2). Based on the topology of the phylogenetic trees, two genotypes of *Ehrlichia* with different haplotypes appear in all processed pools; these are referred to as *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra. The genotype Serrana was identified in 13 pools (86.7%), and the genotype Laguna Negra in 2 (13.3%) positive pools of ticks. The sequences generated in this study were deposited in the GenBank database and the accession numbers are listed in Table 2. The percent nucleotide identities among *16S* rDNA, *dsb* and *groEL* sequences between *Ehrlichia* sp. genotype Serrana, *Ehrlichia* sp. genotype Laguna Negra and the most similar sequence available on GenBank, *Ehrlichia* sp. Magellanica (MK049840, MK049838, MK049839), are shown in Tables 3–5.

4. Discussion

In the present study, we performed molecular screening of *Ehrlichia* spp. in *I. auritulus* and revealed the occurrence of two genotypes of *Ehrlichia* provisionally named Serrana and Laguna Negra. Genetic comparisons conducted for the three studied genes are consistent to consider them as separate organisms. This assumption was confirmed by the topologies of phylogenetic trees since Serrana and Laguna Negra genotypes formed well-defined and separated clades within the genus *Ehrlichia*. Interestingly, *Ehrlichia* sp. Magellanica seems to correspond to a closely related species, because it grouped with both genotypes in all phylogenies. In addition, *Ehrlichia* sp. Magellanica and both genotypes herein characterized were similar when performing nucleotide pairwise comparisons, and all three were detected in ticks that parasitize birds.

In the Southern Cone of South America, the majority of the *Ehrlichia* spp. detected in ticks have been found in ticks from the genera *Amblyomma* and *Rhipicephalus*. Currently, in this region of the American continent, species such as *E. canis* and *E. minasensis* are of medical and veterinary concern (Aguiar, 2017). A remarkable exception is *Ehrlichia* sp. Magellanica that was detected in *I. uriae* in Southern Chile (Muñoz-Leal et al., 2019).

Considering the phylogenetic relationships between these genotypes and their common eco–epidemiological profiles, it is reasonable to state that these species may correspond to a natural lineage associated with birds.

Some *Ehrlichia* species are considered pathogenic for humans and domestic animals, and currently mammals are the only group of vertebrates demonstrated to sustain infections in nature (Rar & Golovljova, 2011; Gofton et al., 2018). However, molecular evidence for a group of ehrlichiae that may infect Magellanic penguins (*S. magellanicus*) was reported in Chile (Muñoz-Leal et al., 2019), and sequences of *16S* rRNA gene related to *E. chaffeensis* were retrieved from a song thrush (*Turdus philomelos*) in Hungary (Hornok et al., 2020). In this study, we detected *Ehrlichia* DNA in *I. auritulus*, a tick that feeds chiefly on birds (Nava et al., 2017; Guglielmone et al., 2020), reinforcing the hypothesis that avian hosts should be evaluated as competent hosts for bacteria of this genus.

Finally, our results constitute the first characterization of *Ehrlichia* spp. in Uruguay. Future studies on birds reported as hosts for *I. auritulus* are needed to gain epidemiological data on both *Ehrlichia* genotypes in the country. Since *I. auritulus* does not parasitize humans, these putatively novel *Ehrlichia* species are probably not involved in human ehrlichiosis.

CRediT author statement

María L. Félix: Conceptualization, Data Curation, Methodology, Formal Analysis, Investigation, Project Administration, Writing - Original Draft, Writing - Review & Editing. Sebastián Muñoz-Leal: Conceptualization, Formal Analysis, Methodology, Writing - Original Draft, Writing - Review & Editing. Luis A. Carvalho: Formal Analysis, Investigation, Methodology, Writing - Review & Editing. Diego Queirolo: Investigation, Methodology, Writing - Review & Editing. Susana Remesar Alonso: Investigation, Methodology, Writing - Review & Editing. Santiago Nava: Formal Analysis, Writing - Original Draft, Writing - Review. María T. Armúa-Fernández: Conceptualization, Data Curation, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. José M. Venzal: Conceptualization, Data Curation,

Methodology, Resources, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. All authors read and approved the final manuscript.

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Declaration of competing interests

The authors declare that they have no competing interests.

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