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Original article

Novel *Ehrlichia* sp. detected in Magellanic penguins (*Sphenicus magellanicus*) and in the seabird tick *Ixodes uriae* from Magdalena Island, southern Chile

Sebastián Muñoz-Leal[a,*], Yara S. Clemes[a], Marcos G. Lopes[a], Igor C.L. Acosta[a,b], Maria Carolina A. Serpa[a], Luis Felipe S.P. Mayorga[c], Solange M. Gennari[a], Daniel González-Acuña[d], Marcelo B. Labruna[a]

[a] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva, 87, Cidade Universitária, São Paulo, SP, 05508-270, Brazil
[b] RRDM – FURG - Programa de Monitoramento da Biodiversidade Aquática na Área Ambiental I - Av. Itália km 8 S/N, Campus Carreiros, 96203-900, Rio Grande, RS, Brazil
[c] Instituto de Pesquisa e Reabilitação de Animais Marinhos (IPRAM), Rodovia BR 262 Km 0 sem numero, CEP 29140130, Cariacica, ES, Brazil
[d] Laboratorio de Parásitos y Enfermedades de Fauna Silvestre, Departamento de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad de Concepción, Av. Vicente Méndez 595, Chillán, Chile

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ABSTRACT

Ehrlichia spp. are obligatory intracellular microorganisms that infect hematopoietic, endothelial or blood cells of mammals. Ticks are the only vectors of these agents in nature. To date, the role of birds and their associated ticks as reservoirs of ehrlichiae remains almost unexplored. In this study, we performed a molecular screening for bacteria of Anaplasmataceae family in samples of spleen (n = 72) and lung (n = 17), recovered from 72 carcasses of Magellanic penguins (*Spheniscus magellanicus*) in Brazil and Chile. One apparently unengorged tick (*Ixodes uriae*) was also collected while wandering upon one of the carcasses and submitted to molecular analyses as well. Through conventional and nested PCR protocols three genes (16S rRNA, *dsb* and *groEL*) of a new *Ehrlichia* sp. were partially characterized upon organs of three penguins and in the tick coming from Magdalena Island (Chile). First matches after BLASTn comparisons showed that our sequences share 99.4% (16S rRNA), 94.6% (*groEL*) and 79.3% (*dsb*) of identity with "*Candidatus* Ehrlichia ornithorhynchi", *Ehrlichia* sp. NS101 and *Ehrlichia canis* CCZ, respectively. Matrixes of genetic distance including other representatives of the *Ehrlichia* genus point a 99.4%, 94.0%, and 80.0% of identity with 16S rRNA, *groEL* and *dsb* genes from *Ehrlichia* sp. It25, *Ehrlichia* sp. NS101, and *Ehrlichia chaffeensis* San Louis, respectively. A Bayesian phylogenetic analysis of Anaplasmataceae 16S rRNA gene places the detected *Ehrlichia* sp. into a group with *Ehrlichia* sp. BAT and *Ehrlichia* sp. Natal. Although depicting different topologies, Bayesian unrooted phylogenetic trees constructed for *groEL* and *dsb* genes position this *Ehrlichia* sp. into well-supported branches, which reinforces the finding of a new taxon. For the moment, any pathogenic effect of this new *Ehrlichia* sp. on penguins is still unknown. However, this fact becomes important to assess from a conservation point of view since populations of Magellanic penguins are currently threatened and in an ongoing decrease.

1. Introduction

The genus *Ehrlichia* is composed by small α-proteobacteria that naturally infect hematopoietic, endothelial, or blood cells of mammals and use ticks as vectors (Kersters et al., 2006). Six species are currently recognized (Parte, 2018), some of them causing severe disease when transmitted to naïve hosts, such as domestic animals and humans (Rar and Golovljova, 2011). Based on molecular techniques, contemporary research has unveiled the occurrence of further genetic variants, and novel species such as "*Candidatus* Ehrlichia shimanensis" (Kawahara et al., 2006), "*Candidatus* Ehrlichia khabarensis" (Rar et al., 2015), "*Candidatus* Ehrlichia occidentalis" (Gofton et al., 2017), and "*Candidatus* Ehrlichia ornithorhynchi" (Gofton et al., 2018) have been proposed. It is evident then that the specific richness of this genus might be much underestimated.

Mammals constitute so far the only competent hosts and reservoirs for ehrlichiae in nature (Rar and Golovljova, 2011; Gofton et al., 2018). However, several findings point that bacteria of this genus would

* Corresponding author.
E-mail address: munoz-leal@usp.br (S. Muñoz-Leal).

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naturally infect a wider array of vertebrate and invertebrate hosts. In fact, *Ehrlichia* DNA has been detected in ticks associated with reptiles (Andoh et al., 2015; Muñoz-Leal et al., 2019) and also in reptiles (Tijsse-Klasen et al., 2010). A striking and open-to-question finding corresponds to the detection of ehrlichiae commonly associated with ungulates and carnivores in blood of wild birds in Brazil (Machado et al., 2012). Reinforcing the role of birds as hosts for haemotropic Anaplasmataceae, a new *Candidatus* taxon belonging to *Ehrlichia*'s sister genus *Anaplasma* was recently characterized from blood of South African penguins (*Spheniscus demersus*) (Vanstreels et al., 2018).

Magellanic penguins (*Spheniscus magellanicus*) reproduce in southern shores of Argentina and Chile during austral summer, and swim towards northern latitudes during austral winter in search for food in warmer conditions (Boersma et al., 2013). The current conservation status of the Magellanic penguin is of concern. By-catch during commercial fishery and oiling incidents correspond to the main threats affecting their populations (BirdLife International, 2018).

Like many seabird species that breed in Subantarctic and Antarctic latitudes, penguins are often parasitized by ticks. In particular, a common hard tick associated with these birds in austral ecosystems corresponds to *Ixodes uriae* (Ixodidae) (Muñoz-Leal and González-Acuña, 2015). While this hard tick can act as a vector for some viruses and spirochetes, bacteria of Anaplasmataceae family have never been reported on it (Muñoz-Leal and González-Acuña, 2015).

In an attempt to contribute with the knowledge on the natural Anaplasmataceae harbored by seabirds, in this study, carcasses of Magellanic penguins obtained from different sources and a tick were PCR-screened for bacteria of this family. Our results describe the detection and molecular characterization of a novel *Ehrlichia* sp.

2. Materials and methods

2.1. Collection of samples

During the years 2014 and 2015, 54 necropsies were performed on Magellanic penguins that perished after stranding lesions or further complications while hosted in the "Centro de Reabilitação de Animais Marinhos do Espírito Santo (CRAM-ES)," municipality of Cariacica, state of Espírito Santo, Brazil (20°19′53″S, 40°21′34″W). In addition, 18 necropsies were performed *in situ* during January 2016 on conspecific dead penguins scattered among the colony that yearly breeds at Magdalena Island, Magallanes' Strait, "Región de Magallanes y de la Antártica Chilena," in Southern Chile (55°55′08″S; 70°34′37″W) (Fig. 1A). Fragments of ≈1 cm^2 of spleen and/or lung were collected from each necropsied specimen in 1.5 mL plastic tubes and stored at -20 °C until processing in the laboratory. While samples of spleen were collected from all 72 penguins, lung fragments were obtained only from 17 birds in Chile. In addition, one apparently unengorged hard tick was collected while walking upon the feathers of a dead specimen at Magdalena Island (Fig. 1B), and placed in a vial with 100% ethanol. The key from Nava et al. (2017) was used to identify this tick by external morphology. In Brazil, all procedures were authorized by local authorities (99/2011-DFyFS-SRRN, N° 083 SsCyAP/12) and approved by the Animal Ethics Committee of the University of São Paulo (CEUA-USP 601415). Necropsies of penguins performed at Magdalena Island were authorized by permits 2799 and 039/2016 given by the "Subsecretaria de Pesca y Acuicultura (SUBPESCA)" and by the "Corporación Nacional Forestal (CONAF)," respectively.

2.2. Molecular analyses

Spleen and/or lung samples collected from penguins in Brazil and Chile, and the entire tick were submitted to DNA extraction using the Wizard Genomic DNA Purification Kit Protocol (Promega Corporation, Madison, WI), following manufacturer's instructions. Successful DNA extractions for penguins and the single tick were confirmed by a conventional PCR targeting penguin mitochondrial hypervariable region I and tick mitochondrial 16S rRNA genes, respectively. In order to determine Anaplasmataceae-positive samples, a conventional PCR targeting the Anaplasmataceae 16S rRNA gene was performed. Positive samples were submitted to different *Ehrlichia*-specific conventional and nested PCR protocols in order to amplify fragments of *groEL* (60 kDa chaperonin) and *dsb* (disulfide oxireductase) genes. All primers used in this study are listed in Table 1. A mix of 25 µl (12.5 µl of DreamTaq Green PCR master mix [2X, Thermo Fischer Scientific Baltics UAB, Vilnius, Lithuania], 1 µl of each primer [10 pmol/µl], 8 µl of ultrapure water and 2.5 µl of template DNA) was prepared to conduct conventional PCRs. Nested rounds were pipetted in a separated room and performed using 1 µl of DNA from the first reaction and 9.5 µl of ultrapure water. All reactions ran without positive controls and included ultrapure water as negative controls. PCR products were resolved in 1.5% agarose gels and visualized through UV-transillumination. Anaplasmataceae and tick mitochondrial 16S rRNA products were treated with ExoSAP-IT (Affimetryx/Thermo Fisher Scientific, Santa Clara, CA), prepared for sequencing with Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and sequenced in an ABI 3500 automatic device (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA). Obtained sequences were assembled with Geneious R9 (Kearse et al., 2012). BLASTn analyses (www.ncbi.nlm.nih.gov/blast) were performed in order to infer closest identities with organisms available in GenBank (Altschul et al., 1990).

2.3. Phylogenetic analyses

Obtained sequences for 16S rRNA, *groEL*, and *dsb* genes were individually aligned using CLUSTAL W (Thompson et al., 1994) with 37, 35, and 27 homologous GenBank-retrieved sequences, respectively. Divergences between different *Ehrlichia* spp. were assessed by visually reading heatmap matrixes of genetic identity constructed for each alignment with Geneious R9.

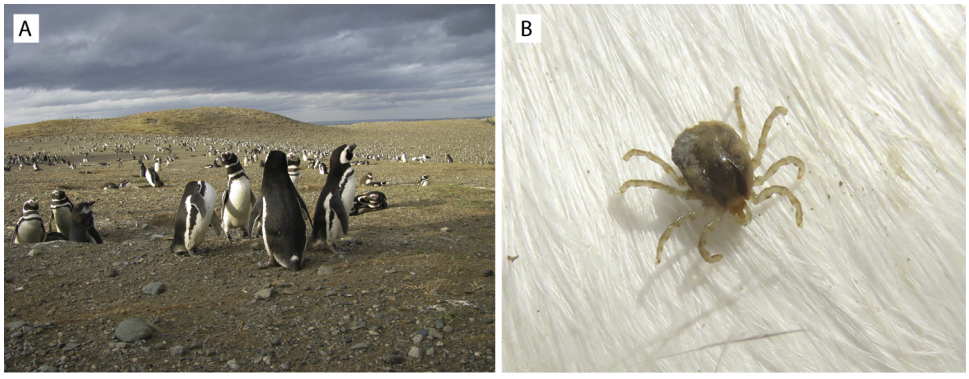

Fig. 1. *In situ* photographs from in Magdalena Island: (A) View of Magellanic penguin colony; (B) Wandering tick collected upon the feathers of one dead penguin.

Table 1
Primers used in conventional and nested PCR.

Targeted gene	Primer name	Sequence (5' to 3')	Reference	PCR protocol (primer forward/ primer reverse): amplicon size
Mitochondrial hypervariable region I	L-tRNAGlu	CCTGCTTGGCTTTTYTCCAAGACC	Roeder et al. (2002)	Conventional PCR (L-tRNAGlu/H-Dbox): 654 bp
	H-Dbox	CTGACCGAGGAACCAGAGGCGC	Roeder et al. (2002)	
16S mitochondrial rRNA	16S + 1	CCGGTCTGAACTCAGATCAAGT	Mangold et al. (1998)	Conventional PCR (16S + 1/16S-1): ≈ 460 bp
	16S-1	GCTCAATGATTTTTTAAATTGCTG	Mangold et al. (1998)	
16S rRNA Anaplasmataceae	EHR16SD*	GGTACCYACAGAAGAAGTCC	Inokuma et al. (2000)	Conventional PCR (EHR16SD/EHR16SR): 346 bp
	EHR16SR*	TAGCACTCATCGTTTACAGC	Inokuma et al. (2000)	
groEL	GR0607F	AAGATGCWGTWGGWTGTACKGC	Takano et al. (2009)	1st round PCR (GR0607 F/GR01294R): 688 bp
	GR01294R	AGMGCTTCWCCTTCWACRTCYTC	Takano et al. (2009)	2nd round PCR (GR0677 F/GR01121R): 445 bp
	GR0677F	ATTACTCAGAGTGCTTCTCARTG	Takano et al. (2009)	
	GR01121R	TGCATACCRTCAGTYTTTTCAAC	Takano et al. (2009)	
dsb	DSB-330	GATGATGTCTGAAGATATGAAACAAAT	Doyle et al. (2005)	1st round PCR (DSB-330/DSB-728): 401 bp
	DSB-728	CTGCTCGTCTATTTTACTTCTTAAAGT	Doyle et al. (2005)	2nd round PCR (DSB-380/DSB-728): 349 bp
	DSB-380	ATTTTTAGRGATTTTCCAATACTTGG	Almeida et al. (2013)	

* primers used in the initial PCR screening.

Sequences of *Anaplasma* spp. (U02521; AY048816) were used as out-group for Anaplasmataceae 16S rRNA gene phylogeny. Not intending to infer evolutionary relationships but rather to depict whether the position of the sequences resulted in well-defined branches or not, unrooted phylogenetic trees were constructed for *groEL* and *dsb* genes. Nucleotide substitution models were calculated with MEGA 5 (Tamura et al., 2011). While general time reversible (GTR) with invariable gamma distribution model was used to obtain Anaplasmataceae 16S rDNA phylogeny, we employed the GTR model combined with gamma distribution to run *groEL* and *dsb* trees. Phylogenies were constructed using the Bayesian method implemented in Mrbayes 3.2.5 software (Huelsenbeck and Ronquist, 2001) with 1,000,000 generations; each tree was sampled every 1000 generations, ran four times and began with random starting trees. In all phylogenetic inferences, the first 25% of the trees represented burn-in, and the remaining subset of trees were used to calculate the Bayesian posterior probability.

3. Results

3.1. Positive samples and BLASTn comparisons

All spleen and lung extractions were positive for penguin mitochondrial hypervariable region I PCR, and the single collected tick yielded positive amplicons for mitochondrial 16S rRNA PCR. Only three spleen samples, each obtained from one penguin at Magdalena Island were positive for Anaplasmataceae 16S rRNA, *groEL*, and *dsb* genes; lung samples from two out of these three penguins yielded the same PCR results. The sole collected tick was identified as a female of *I. uriae* by external morphology. This diagnosis was confirmed by sequencing a partial fragment of 16S rRNA gene that showed 98.52% of identity (402/408 bp, 1 gap, 100% query cover, 0.0 E-value) with *I. uriae* from Sweden (AB087746) and 97.31% identical (398/409 bp, 2 gaps, 100% query cover, 0.0 E-value) with conspecific ticks from the Antarctic Peninsula (MH183257). Remarkably, the female tick was positive to all three Anaplasmataceae loci as well. Sequences obtained from penguin organs were identical to sequences retrieved from the tick for each of the three genes. After BLASTn comparisons, first matches for homologous sequences in GenBank were as follows: 1) Anaplasmataceae 16S rRNA gene: 99.4% identical (345/347 bp, 99% query cover, 0 gaps, 8e-177 E-value) with the sequence of "*Ca*. E. ornithorhynchi" (MF069159); 2) *dsb* gene: 79.3% identical (279/352 bp, 97% query cover, 7 gaps, 6e-59 E-value) to *E. canis* CCZ (MG772657); and 3) *groEL* gene: 94.6% of identity (316/334 bp, 99% query cover, 0 gaps, 6e-143 E-value) with *Ehrlichia* sp. NS101 (AB454077). GenBank accession numbers for the sequences generated in this study are: MK049838 (*dsb*), MK049839 (*groEL*), MK049840 (*Ehrlichia* sp. 16S rRNA), and MK570083 (tick mitochondrial 16S rRNA gene).

3.2. Genetic identities and phylogenetic analyses

Compared with other representatives of the genus *Ehrlichia*, percentages of genetic identity calculated for each of the three constructed alignments point that the *Ehrlichia* sp. detected in the present study (now onwards "*Ehrlichia* sp. Magellanica") showed identities (minimum–maximum) of 96.8–99.4%, 84.5–94.0% and 75.8–80.0% for 16S rRNA, *groEL* and *dsb* genes, respectively. Moreover, compared with valid species of the genus (*E. canis*, *E. chaffeensis*, *E. ewingii*, *E. minasensis*, *E. muris*, and *E. ruminantium*), and with four *Candidatus* taxa ("*Ca*. E. shimanesis", "*Ca*. E. occidentalis", "*Ca*. E. ornithorhynchi" and "*Ca*. E. khabarensis"), the highest nucleotide similarities for each of the three characterized loci of *Ehrlichia* sp. Magellanica were always below the minimum level of intraspecific genetic similarities for each of these species (Tables S1, S2, and S3).

Phylogenetic analysis of Anaplasmataceae 16S rRNA gene clearly shows our sequence belonging to the *Ehrlichia* genus (Fig. 2A). The 16S rRNA tree indicates that *Ehrlichia* sp. Magellanica forms a polytomic clade with *Ehrlichia* sp. BAT and *Ehrlichia* sp. Natal, the former detected in the bat-tick *Carios vespertilionis* in France (Socolovschi et al., 2012) and the later characterized from an opossum's spleen in Brazil (Lopes et al., 2018). On the other hand, unrooted *dsb* and *groEL* phylogenies show *Ehrlichia* sp. Magellanica sequences in two different positions within the genus. Although both analyses differ in their topologies, they coincide in placing *Ehrlichia* sp. Magellanica into well-supported branches, thus confirming a new genetic variant (Fig. 2B, C).

4. Discussion

Magellanic penguins are South American migratory birds that seasonally shift their populations between Brazilian shores and southern territories from Argentina and Chile, where they reproduce during austral summer (Boersma et al., 2013). Although in this study we included samples from both populations, only penguins from Magdalena Island were positive to *Ehrlichia* PCR targeting 16S rRNA, *dsb,* and *groEL* genes. Such a result indicating that infected penguins occur only in the southern range of their distribution should be carefully interpreted since we analyzed a limited number of specimens from both localities.

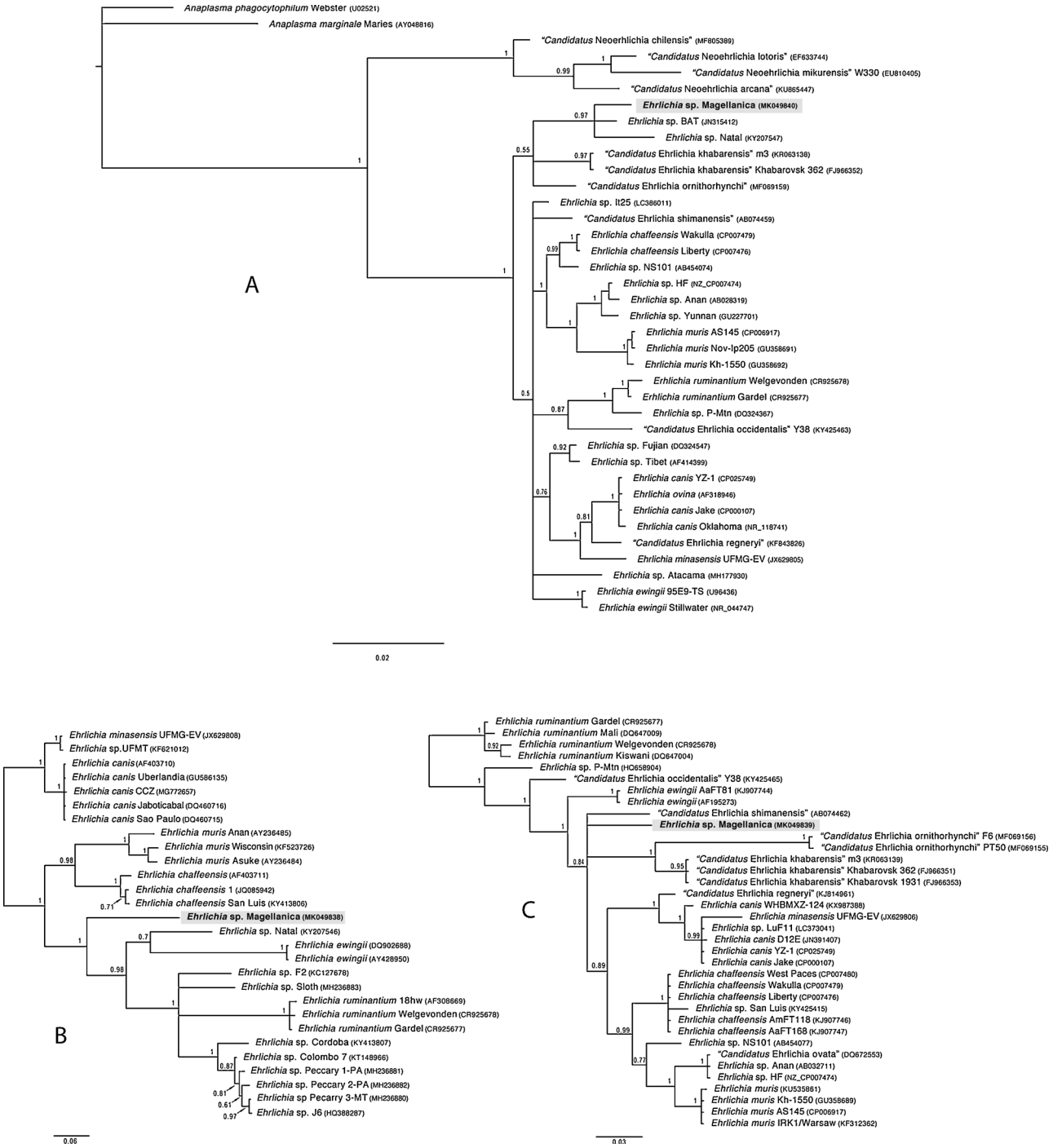

Fig. 2. Bayesian phylogenetic analyses inferred for partial fragments of 350 bp, 354 bp and 335 bp of the genes (A) 16S rRNA, (B) *dsb*, and (C) *groEL*, respectively. Bayesian posterior probabilities are indicated upon or arrowing each branch. The position of *Ehrlichia* sp. Magellanica is highlighted within a grey rectangle. Scale bar indicates the number of substitutions per nucleotide position.

Noteworthy, identical sequences for all three loci were retrieved from an apparently unengorged female of *I. uriae* collected in Magdalena Island as well. Although this fact suggests that this tick species may correspond to the vector of this agent, studies on vector competence are needed before reaching any conclusion. Notwithstanding, *Ixodes* spp. have already been recognized as vectors of *Ehrlichia* spp. in other ecosystems (Shibata et al., 2000; Pritt et al., 2017), a fact that reinforces this hypothesis. As in the Southern Hemisphere *I. uriae* parasitizes seabird in Subantarctic and Antarctic lands (Muñoz-Leal and González-Acuña, 2015), infected ticks in Magdalena Island could directly increase the probability to find a positive bird, which would explain the results of our study.

Last decade research on Anaplasmataceae bacteria has put in evidence that the diversity of the genus *Ehrlichia* is underestimated (Rar et al., 2015; Gofton et al., 2017, 2018). Although in the current study we characterized relatively short sequences of 16S rRNA, *dsb,* and *groEL* genes, calculated percentages of interspecific genetic identities support that *Ehrlichia* sp. Magellanica correspond to a novel genetic variant (Tables S1, S2, S3). In particular, a 99.4% of identity (i. e., 2 bp-difference) between *Ehrlichia* sp. Magellanica and *Ehrlichia* sp. It25 was observed after comparisons of a 350 bp fragment of 16S rRNA gene, which could be suggestive of conspecificity. However, given the conservative

nature of this gene and the shortness of our analysis, ≥99.4% values were also observed between well-accepted species within the genus (Table S1). Therefore, a definitive conclusion on the identity of *Ehrlichia* sp. Magellanica based on 16S rRNA gene must rely on the analysis of longer sequences. On the other hand, unlike other intracellular microorganisms transmitted by ticks (i. e. *Rickettsia* spp. [Raoult et al., 2005]), an algorithm for separating species based on genetic identities for a set of genes still lacks for the *Ehrlichia* genus. The discovery and further characterization of novel variants of ehrlichiae should contribute to the construction of such a classification in the future.

Phylogenetic uncertainty in the form of polytomies is expectable when performing phylogenetic analyses whit short nucleotide sequences, as depicted in the topologies of all three phylogenetic trees herein obtained. Despite this limitation, phylogenetic analyses support that obtained Anaplasmataceae 16S rRNA sequences do belong to *Ehrlichia* genus. In fact, although *Ehrlichia* sp. Magellanica forms a polytomic group with *Ehrlichia* sp. BAT and *Erhlichia* sp. Natal, two genetic variants that also need further molecular characterization, the trichotomy is well supported (0.97, Fig. 2A). Additional evidence for a novel variant can be inferred by observing the topologies of unrooted *dsb* and *groEL* phylogenies, in which *Ehrlichia* sp. Magellanica appears as a well-defined evolutionary lineage within the genus under high Bayesian posterior probabilities values (Fig. 2B, C).

Discounting some exceptions that suggest reptiles and reptile-ticks as possible hosts for ehrlichiae (Tijsse-Klasen et al., 2010; Andoh et al., 2015; Muñoz-Leal et al., 2019), mammals have been the sole vertebrate group demonstrated to sustain *Ehrlichia* infections in nature (Rar and Golovljova, 2011; Gofton et al., 2018). Remarkably, the evidence compiled in the current study points that ehrlichiae also infect birds and might be transmitted by avian ticks. Noteworthy, a similar ecologic scenario for an haemotropic Anaplasmataceae was recently reported in South African penguins, yet in this case the vector remains unknown (Vanstreels et al., 2018). These discoveries underline the role of seabirds and their ticks as reservoirs for Anaplasmataceae bacteria, and give place to questions on an incipient diversity of these microorganisms that could be possibly associated with this niche. For instance, an *Anaplasma*-like microorganism has been detected in *Ornithodoros spheniscus*, another penguin-associated tick (Muñoz-Leal et al., 2019).

Finally, Anaplasmataceae bacteria infecting wildlife generally cause asymptomatic infections (Rar and Golovljova, 2011; Gofton et al., 2018). Considering this, we are unaware if penguins from Magdalena Island that were positive to *Ehrlichia* sp. Magellanica perished because of the infection with this bacterium. However, a pathogenic effect triggered by the exposure of these birds to a stressful milieu should not be overlooked. This caveat becomes particularly important from a conservation viewpoint, since populations of Magellanic penguins are currently decreasing (BirdLife International, 2018).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.06.015.

References

Almeida, A.P., Souza, T.D., Marcili, A., Labruna, M.B., 2013. Novel *Ehrlichia* and *Hepatozoon* agents infecting the Crab-Eating fox (*Cerdocyon thous*) in Southeastern Brazil. J. Med. Entomol. 50, 640–646. https://doi.org/10.1603/ME12272.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Andoh, M., Sakata, A., Takano, A., Kawabata, H., Fujita, H., Une, Y., Goka, K., Kishimoto, T., Ando, S., 2015. Detection of *Rickettsia* and *Ehrlichia* spp. in ticks associated with exotic reptiles and amphibians imported into Japan. PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0133700.

BirdLife International, 2018. Spheniscus magellanicus. IUCN Red List Threat. https://doi.org/10.1007/978-1-349-02270-0_11. Species e.T2269782.

Boersma, P.D., Frere, E., Kane, O., Pozzi, L.M., Pütz, K., Rey, A.R., Rebstock, G.A., Simeone, A., Smith, J., Bureen, A.V., Yorio, P., García, P.B., 2013. Magellanic penguins (*Spheniscus magellanicus*). In: García, P.B., Boersma, P.D. (Eds.), Penguins Natural history and conservation. University of Washington Press, pp. 328.

Doyle, C.K., Labruna, M.B., Breitschwerdt, E.B., Tang, Y., Corstvet, R.E., Hegarty, B.C., Bloch, K.C., Li, P., Walker, D.H., Mcbride, J.W., 2005. Detection of medically important *Ehrlichia* by quantitative multicolor TaqMan Real-Time Polymerase Chain Reaction of the *dsb* gene. JMD 7, 504–510.

Gofton, A.W., Loh, S.M., Barbosa, A.D., Paparini, A., Gillett, A., Macgregor, J., Oskam, C.L., Ryan, U.M., Irwin, P.J., 2018. A novel *Ehrlichia* species in blood and *Ixodes ornithorhynchi* ticks from platypuses (*Ornithorhynchus anatinus*) in Queensland and Tasmania, Australia. Ticks Tick. Dis. 9, 435–442. https://doi.org/10.1016/j.ttbdis.2017.12.011.

Gofton, A.W., Waudby, H.P., Petit, S., Greay, T.L., Ryan, U.M., Irwin, P.J., 2017. Ticks and Tick-borne diseases detection and phylogenetic characterisation of novel *Anaplasma* and *Ehrlichia* species in *Amblyomma triguttatum* subsp. from four allopatric populations in Australia. Ticks Tick. Dis. 8, 749–756. https://doi.org/10.1016/j.ttbdis.2017.05.009.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755. https://doi.org/10.1093/bioinformatics/17.8.754.

Inokuma, H., Raoult, D., Brouqui, P., 2000. Detection of *Ehrlichia platys*DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. J. Clin. Microbiol. 38, 4219–4221.

Kawahara, M., Rikihisa, Y., Lin, Q., Isogai, E., Tahara, K., Itagaki, A., Hiramitsu, Y., Tajima, T., 2006. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Appl. Environ. Microbiol. 72, 1102–1109. https://doi.org/10.1128/AEM.72.2.1102.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., Stackebrandt, E., 2006. Introduction to the Proteobacteria. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandr, E. (Eds.), The Prokaryotes. A Handbook an the Biology of Bacteria. Volume 5: Proteobacteria: Alpha and Beta Subclasses, https://doi.org/10.1007/0-387-30741-9.

Lopes, M.G., Muñoz-Leal, S., de Lima, J.T.R., Fournier, G.Fda S.R., Acosta, Ida C.L., Martins, T.F., Ramirez, D.G., Gennari, S.M., Labruna, M.B., 2018. Ticks, rickettsial and erlichial infection in small mammals from Atlantic forest remnants in northeastern Brazil. Int. J. Parasitol. Parasites Wildl. 7, 380–385. https://doi.org/10.1016/j.ijppaw.2018.10.001.

Machado, R.Z., André, M.R., Werther, K., de Sousa, E., Gavioli, F.A., Alves Junior, J.R.F., 2012. Migratory and carnivorous birds in Brazil: reservoirs for *Anaplasma* and *Ehrlichia* species? Vector-Borne Zoonotic Dis. 12, 705–708. https://doi.org/10.1089/vbz.2011.0803.

Mangold, A.J., Bargues, M.D., Mas-Coma, S., 1998. Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). Parasitol. Res. 84, 478–484.

Muñoz-Leal, S., González-Acuña, D., 2015. The tick *Ixodes uriae* (Acari: Ixodidae): hosts, geographical distribution, and vector roles. Ticks Tick. Dis. 6, 843–868. https://doi.org/10.1016/j.ttbdis.2015.07.014.

Muñoz-Leal, S., Lopes, M.G., Marcili, A., Martins, T.F., González-Acuña, D., Labruna, M.B., 2019. Anaplasmataceae, *Borrelia* and *Hepatozoon* agents in ticks (Acari: Argasidae, Ixodidae) from Chile. Acta Trop. 192, 91–103. https://doi.org/10.1016/j.actatropica.2019.02.002.

Nava, S., Venzal, J.M., González-Acuña, D., Martins, T.F., Guglielmone, A.A., 2017. Ticks of the Southern Cone of America: Diagnosis, Distribution and Hosts with Taxonomy, Ecology and Sanitary Importance. Elsevier, Academic Press.

Parte, A.C., 2018. LPSN - list of prokaryotic names with standing in nomenclature (Bacterio.net), 20 years on. Int. J. Syst. Evol. Microbiol. 68, 1825–1829. https://doi.org/10.1099/ijsem.0.002786.

Pritt, B.S., Allerdice, M.E.J., Sloan, L.M., Paddock, C.D., Munderloh, U.G., Rikihisa, Y., Tajima, T., Paskewitz, S.M., Neitzel, D.F., Hoang Johnson, D.K., Schiffman, E., Davis, J.P., Goldsmith, C.S., Nelson, C.M., Karpathy, S.E., 2017. Proposal to reclassify *Ehrlichia muris* as *Ehrlichia muris* subsp. *muris*subsp. nov. and description of *Ehrlichia muris* subsp. *eauclairensis* subsp. nov., a newly recognized tick-borne pathogen of humans. Int. J. Syst. Evol. Microbiol. 67, 2121–2126. https://doi.org/10.1099/ijsem.0.001896.

Raoult, D., Fournier, P.E., Eremeeva, M., Graves, S., Kelly, P.J., Oteo, J., Sekeyova, Z., Tamura, A., Tarasevich, I., Zhang, L., 2005. Naming of rickettsiae and rickettsial diseases. Ann. N. Y. Acad. Sci. 1063, 1–12. https://doi.org/10.1196/annals.1355.002.

Rar, V.A., Pukhovskaya, N.M., Ryabchikova, E.I., Vysochina, N.P., Bakhmetyeva, S.V., Zdanovskaia, N.I., Ivanov, L.I., Tikunova, N.V., 2015. Ticks and tick-borne diseases molecular-genetic and ultrastructural characteristics of "*Candidatus* Ehrlichia khabarensis", a new member of the *Ehrlichia* genus. Ticks Tick. Dis. 6, 658–667. https://doi.org/10.1016/j.ttbdis.2015.05.012.

Rar, V., Golovljova, I., 2011. *Anaplasma*, *Ehrlichia*, and "*Candidatus* Neoehrlichia" bacteria: pathogenicity, biodiversity, and molecular genetic characteristics, a review. Infect. Genet. Evol. 11, 1842–1861. https://doi.org/10.1016/j.meegid.2011.09.019.

Roeder, A.D., Ritchie, P.A., Lambert, D.M., 2002. New DNA markers for penguins. Conserv. Genet. 3, 341–344. https://doi.org/10.1023/A:1019982009059.

Shibata, S., Kawahara, M., Rikihisa, Y., Fujita, H., Watanabe, Y., Suto, C., Ito, T., 2000. New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J. Clin. Microbiol. 38, 1331–1338.

Socolovschi, C., Kernif, T., Raoult, D., Parola, P., 2012. *Borrelia*, *Rickettsia*, and *Ehrlichia* species in bat ticks, France, 2010. Emerg. Infect. Dis. 18, 1966–1975. https://doi.org/10.3201/eid1812.111237.

Takano, A., Ando, S., Kishimoto, T., Fujita, H., Kadosaka, T., Nitta, Y., Kawabata, H., Watanabe, H., 2009. Presence of a novel *Ehrlichia* sp. in *Ixodes granulatus* found in Okinawa, Japan. Microbiol. Immunol. 53, 101–106. https://doi.org/10.1111/j.1348-0421.2008.00093.x.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. https://doi.org/10.1093/molbev/msr121.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1093/nar/22.22.4673.

Tijsse-Klasen, E., Fonville, M., Reimerink, J.H., Spitzen-Van Der Sluijs, A., Sprong, H., 2010. Role of sand lizards in the ecology of Lyme and other tick-borne diseases in the Netherlands. Parasit. Vectors 3, 1–11. https://doi.org/10.1186/1756-3305-3-42.

Vanstreels, R.E.T., Yabsley, M.J., Parsons, N.J., Swanepoel, L., Pistorius, P.A., 2018. A novel candidate species of *Anaplasma* that infects avian erythrocytes. Parasit. Vectors 11, 525. https://doi.org/10.1186/s13071-018-3089-9.