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Characterization of "*Candidatus* Ehrlichia Pampeana" in *Haemaphysalis juxtakochi* Ticks and Gray Brocket Deer (*Mazama gouazoubira*) from Uruguay

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Citation: Félix, M.L.; Muñoz-Leal, S.; Carvalho, L.A.; Queirolo, D.; Remesar, S.; Armúa-Fernández, M.T.; Venzal, J.M. Characterization of "*Candidatus* Ehrlichia Pampeana" in *Haemaphysalis juxtakochi* Ticks and Gray Brocket Deer (*Mazama gouazoubira*) from Uruguay. *Microorganisms* **2021**, *9*, 2165. https://doi.org/10.3390/microorganisms9102165

Academic Editor: Pat Nuttall

Received: 2 September 2021
Accepted: 2 October 2021
Published: 17 October 2021

Abstract: Human ehrlichiosis are scantily documented in Uruguay. The aim of this study was to investigate the presence of *Ehrlichia* spp. in *Haemaphysalis juxtakochi* and in a gray brocket deer (*Mazama gouazoubira*) from Uruguay. The presence of *Ehrlichia* DNA was investigated in free-living *H. juxtakochi* in five localities of southeast and northeast Uruguay, as well as blood, spleen, and ticks retrieved from a *M. gouazoubira*. *Ehrlichia* spp. DNA was detected in six out of 99 tick pools from vegetation, in the spleen of *M. gouazoubira*, and in one out of five pools of ticks feeding on this cervid. Bayesian inference analyses for three loci (*16S* rRNA, *dsb*, and *groEL*) revealed the presence of a new rickettsial organism, named herein as "*Candidatus* Ehrlichia pampeana". This new detected *Ehrlichia* is phylogenetically related to those found in ticks from Asia, as well as *Ehrlichia ewingii* from USA and Cameroon. Although the potential pathogenicity of "*Ca.* E. pampeana" for humans is currently unknown, some eco-epidemiological factors may be relevant to its possible pathogenic role, namely: (i) the phylogenetic closeness with the zoonotic agent *E. ewingii*, (ii) the evidence of *H. juxtakochi* parasitizing humans, and (iii) the importance of cervids as reservoirs for zoonotic *Ehrlichia* spp. The molecular detection of "*Ca.* E. pampeana" represents the third *Ehrlichia* genotype described in Uruguay.

Keywords: Rickettsiales; Anaplasmataceae; *Ehrlichia*; molecular characterization; ticks; *Haemaphysalis juxtakochi*; gray brocket deer; Uruguay

1. Introduction

Ehrlichiae are small Gram-negative tick-transmitted coccobacilli that obligately dwell inside cells. These microorganisms are classified as α-proteobacteria belonging to the family Anaplasmataceae included in the order Rickettsiales [1]. Wild mammals, and probably birds [2], constitute natural vertebrate hosts for *Ehrlichia* spp., which are horizontally transmitted through tick bites [3]. *Ehrlichia* species infect different cells in mammals and ticks. While monocytes, neutrophils, or endothelial cells have been detected as the mammalian target cells, salivary glands, intestinal epithelium, and hemolymph cells, are infected in the vectors [4]. Some *Ehrlichia* spp. exhibit tropism for mononuclear phagocyttablees and compose microcolonies within membrane-bound cytoplasmic vacuoles known as

morulae [5]. These bacteria are the agents of ehrlichiosis, a complex of life-threatening emerging zoonoses and diseases of worldwide veterinary concern [6]. The genus *Ehrlichia* currently consists of six validly published species, namely *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia minasensis*, *Ehrlichia muris*, and *Ehrlichia ruminantium* [7]. For all these species, sequences of the complete chromosome are already available in genetic databases [3]. Four species (*E. chaffeensis*, *E. ewingii*, *E. canis*, and *E. muris*) are known to infect humans and cause potentially severe to fatal ehrlichiosis [7]. Cervids (Cervidae) have been demonstrated to be reservoirs of human pathogenic Ehrlichiae, particularly *E. chaffeensis*, *E. ewingii*, and *E. muris* [8–12].

Recent molecular studies performed in South America have unveiled novel genotypes of *Ehrlichia* retrieved from domestic and wild vertebrates, as well as from their ticks [2,13]. For instance, in Uruguay there is only one characterization of *Ehrlichia* which corresponds to two novel genotypes currently found in *Ixodes auritulus* [14]. There are several studies reporting the evidence of deer as reservoirs of pathogenic *Ehrlichia* spp. in northern latitudes of the globe [9,10]. Despite the report of a case of autochthonous human ehrlichiosis in 2001 [15], this disease in Uruguay has unclear status. The fact that *I. auritulus* does not bite humans [16] prompted us to consider the presence of other tick vectors of *Ehrlichia* spp., and that the disease could be underdiagnosed in this country. Interestingly, *Haemaphysalis juxtakochi* is one of the five species of ticks that have been reported parasitizing humans in Uruguay [16], and cervids are common hosts mainly for its adult stages [17,18]. Therefore, the present study aimed to investigate the presence of *Ehrlichia* spp. in *H. juxtakochi* and its host, the gray brocket deer (*Mazama gouazoubira*) in Uruguay.

2. Materials and Methods

Between March 2014 and August 2017 field work was conducted in five localities of Uruguay: Gruta de los Cuervos (31°37′08″ S, 56°02′47″ W), Tacuarembó Department; Amarillo (31°39′49″ S, 55°03′02″ W) and Lunarejo (31°08′29″ S, 55°54′01″ W), Rivera Department; Reserva Natural Salus (34°25′16″ S, 55°18′54″ W), Lavalleja Department; and Laguna Negra (34°05′09″ S, 53°44′17″ W), Rocha Department.

Ticks were collected from vegetation using the flagging method as described previously [14] and stored in plastic containers with 95% ethanol. In addition, a juvenile female *M. gouazoubira* carcass killed by dogs in September 2017 at Gruta de los Cuervos, Tacuarembó Department was included in this study. Ticks and a sample from the spleen and blood were retrieved from the carcass and stored at −20 °C until use. Ticks were identified using a Nikon stereo microscope SMZ1000 following morphological keys for larval, nymph, and adult stages [17,19]. Since *Ehrlichia* species are not maintained by transovarial transmission [20], only nymphs and adult ticks were analyzed in this study. Ticks were pooled according to sex, developmental stage, site, collection date, and host. Ticks were rinsed with distilled water to remove ethanol, and the ticks were cut thoroughly with dissecting scissors. DNA was extracted using a GeneJET Genomic DNA Purification kit (Thermo Scientific, Vilnius, Lithuania), according to manufacturer's instructions. DNA concentration was estimated using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

For *Ehrlichia* DNA detection, a molecular screening targeting a fragment of the *16S* rRNA gene of the Anaplasmataceae family was carried out using primers and protocols as described previously [21]. Subsequently, positive samples were subjected to two additional PCR protocols to amplify a nearly full-length sequence (~1431 bp) of the *16S* rRNA gene based on two overlapping fragments [22,23]. In addition, a nested and a heminested PCR targeting partial fragments of *groEL* (60 kDa chaperonin) and *dsb* (disulfide oxireductase) genes, respectively, were performed [24–28]. All primers used in this study and fragment sizes are listed in Table 1. Distilled water and DNA of *E. canis* were included as negative and positive controls, respectively. PCR products were analyzed by electrophoresis in 1.5% agarose gels. Amplicons were purified using a GeneJET PCR purification kit (Thermo Fisher Scientific, Vilnius, Lithuania) and sent for sequencing to Macrogen (Seoul, Korea).

BLASTn analyses (www.ncbi.nlm.nih.gov/blast, accessed date: 4 October 2021) were performed in order to infer closest identities with microorganisms available in the GenBank database [29], and to include those sequences in a phylogenetic analysis.

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

Primer Name	Targeted Gene	Sequence (5′–3′)	Amplicon Size (bp)	Reference
EHR16SD *	*16S* rRNA	GGT ACC YAC AGA AGA AGT CC	345	[21]
EHR16SR *		TAG CAC TCA TCG TTT ACA GC		[21]
fD1		AGA GTT TGA TCC TGG CTC AG	~1431	[22,23]
rP2		ACG GCT ACC TTG TTA CGA CTT		[22,23]
HS1a	*groEL*	AIT GGG CTG GTA ITG AAA T	~1400	[24,26]
HS6a		CCI CCI GGI ACI AIA CCT TC		[24,26]
HS43		ATW GCW AAR GAA GCA TAG TC	1297	[25]
HSVR		CTC AAC AGC AGC TCT AGT AGC		[25]
Dsb-330	*dsb*	GAT GAT GTT TGA AGA TAT SAA ACA AAT	401	[27,28]
Dsb-720 **		CTA TTT TAC TTC TTA AAG TTG ATA WAT C	349	[27,28]
Dsb-380		ATT TTT AGR GAT TTT CCA ATA CTT GG		[28]

* Primers used in the initial PCR screening, ** primer used on first and second round.

Phylogenies for the genus *Ehrlichia* were constructed with sequences of each amplified gene and GenBank-retrieved homologues. The alignments for *16S* rRNA, *dsb,* and *groEL* were implemented in CLUSTAL W [30]. We used Bayesian inferences to reconstruct evolutionary relationships in the genus with MrBayes 3.2.5 [31]. The general time reversible (GTR) model was selected to run all the phylogenies using 1,000,000 generations. Each tree was sampled every 100 generations, beginning with random seeds, and ran four times. The first 25% of the trees were considered burn-in, and the remaining trees used to calculate Bayesian posterior probabilities. Sequences of *Neoehrlichia mikurensis* (EU810406; AB213021) and *E. ruminantium* (AF308669) rooted the phylogenetic trees.

3. Results

A total of 5772 *H. juxtakochi* ticks (89 females, 107 males, 1681 nymphs, and 3895 larvae) were collected from vegetation in nineteen samplings carried out in the five selected localities (Table S1). Additionally, 18 *H. juxtakochi* (five females, four males, and nine nymphs) were obtained from the of *M. gouazoubira* carcass.

For *Ehrlichia* DNA detection, 1864 *H. juxtakochi* collected from vegetation (85 females, 98 males, and 1681 nymphs) were divided in 99 samples and analyzed (Table 2). The ticks were processed individually or grouped in pools containing 2 to 62 specimens collected upon the vegetation. The samples examined from *M. gouazoubira* were: five pools containing *H. juxtakochi* ticks (one containing five females, one with four males, and three with three nymphs each) as well as a blood and a spleen sample.

Six out of the 99 *H. juxtakochi* samples containing ticks from the vegetation were positive (6.1%) (4 out of 71 nymph pools, 1 out of 14 male pools, and 1 of 14 female pools) (Table 2). Partial sequences generated for *16S* rRNA, *dsb,* and *groEL* loci of *Ehrlichia* sp. were deposited in GenBank with the accession numbers listed in Table 2. Moreover, one out of the three nymph pools containing *H. juxtakochi* specimens retrieved from *M. gouazoubira* was positive (GenBank accession numbers: MZ733621, MZ779087, and MZ779096 for *16S* rRNA, *dsb,* and *groEL*, respectively). For the samples of blood and spleen of *M. gouazoubira* only a partial sequence for *groEL* of *Ehrlichia* sp. was obtained from the spleen (GenBank accession number MZ779099).

Table 2. Data of *Haemaphysalis juxtakochi* collected in vegetation for each site and processed for detection of "*Candidatus* Ehrlichia pampeana".

Collection Site	Stage	N° of Ticks Processed	Pools	Positive Pools	Positive Pools Code	GenBank Accession Numbers		
						16S rRNA	*dsb*	*groEL*
Gruta de los Cuervos (T)	Female	65	7	1	S16HH13	MZ733618	MZ779092	MZ779098
	Male	77	8	1	S12HM25	-	MZ779089	-
	Nymph	969	29	2	S11HN18 S14HN39	MZ733620 MZ733619	MZ779088 MZ779091	MZ779097 MZ779095
Amarillo (Ri)	Female	0	0	0				
	Male	0	0	0				
	Nymph	12	2	0				
Lunarejo (Ri)	Female	10	3	0				
	Male	15	4	0				
	Nymph	401	15	1	S14HN30	-	MZ779090	MZ779094
Reserva Natural Salus (L)	Female	3	2	0				
	Male	4	1	0				
	Nymph	124	7	0				
Laguna Negra (Ro)	Female	7	2	0				
	Male	2	1	0				
	Nymph	175	18	1	S8HN5	MZ733617	MZ779093	-
Total		1864	99	6				

(T) Tacuarembó, (Ri) Rivera, (L) Lavalleja, (Ro) Rocha.

The comparison among the sequences obtained herein revealed a similarity percentage of 100 and between 99.38–100% and 99.69–100% for *16S* rRNA, *groEL*, and *dsb* fragment genes, respectively. Analyses of the *16S* rRNA sequences retrieved from *H. juxtakochi* (610 to 1234 bp) revealed 100% identity with *Ehrlichia* sp. clone HLAE331 obtained from *Haemaphysalis longicornis* from South Korea (GenBank accession number: GU075697) and 99.92% with two sequences named as *Ehrlichia* sp. TC249-2 and *Ehrlichia* sp. TC251-2 from *Dermacentor nuttalli* from China (KJ410252-KJ410253). Sequences of the *16S* rRNA gene of other *Ehrlichia* spp. obtained from ticks from different parts of the world were <90% identical. Accordingly, partial sequences of *groEL* obtained from *H. juxtakochi* and spleen of *M. gouazoubira* (1140 and 1242 bp, respectively) also showed high identity (97.73–97.91%) with sequences from the *Ehrlichia* sp. detected in *D. nuttalli* from China (KJ410294-KJ410296). In contrast, the closest identity for the *dsb* gene (295 to 330 bp) was *E. ewingii*, detected in *Amblyomma americanum* from USA (AY428950: 90.25%) and from a dog from Cameroon (DQ151999: 90.10%).

The phylogenetic relationship of characterized *Ehrlichia* genes was assessed through Bayesian analyses. Phylogenetic trees constructed with partial sequences of *16S* rRNA and *groEL* produced similar topologies (Figure 1a,c). Although with low support, the *Ehrlichia 16S* rRNA sequences obtained in this study formed a clade with sequences of *Ehrlichia* spp. characterized from *H. longicornis* (HQ697588, MT258398, MT258399) and *Haemaphysalis flava* (MT258401) from Japan, and *D. nuttalli* from China (KJ410251–KJ410253) (Figure 1a). Similarly to the results obtained for the *16S* rRNA gene, the *groEL* sequences formed a clade with sequences of *Ehrlichia* spp. from *Haemaphysalis*, *Dermacentor,* and *Hyalomma* ticks from Asia, as well as *E. ewingii* from a human and *A. americanum* from USA (AF195273, KJ907744) (Figure 1c). In contrast, *dsb* sequences clustered with *E. ewingii* homologues

retrieved from *A. americanum* and *Amblyomma inornatum* from USA (AY428950, KM458249), and *Rhipicephalus sanguineus* from Cameroon (DQ902688) (Figure 1b).

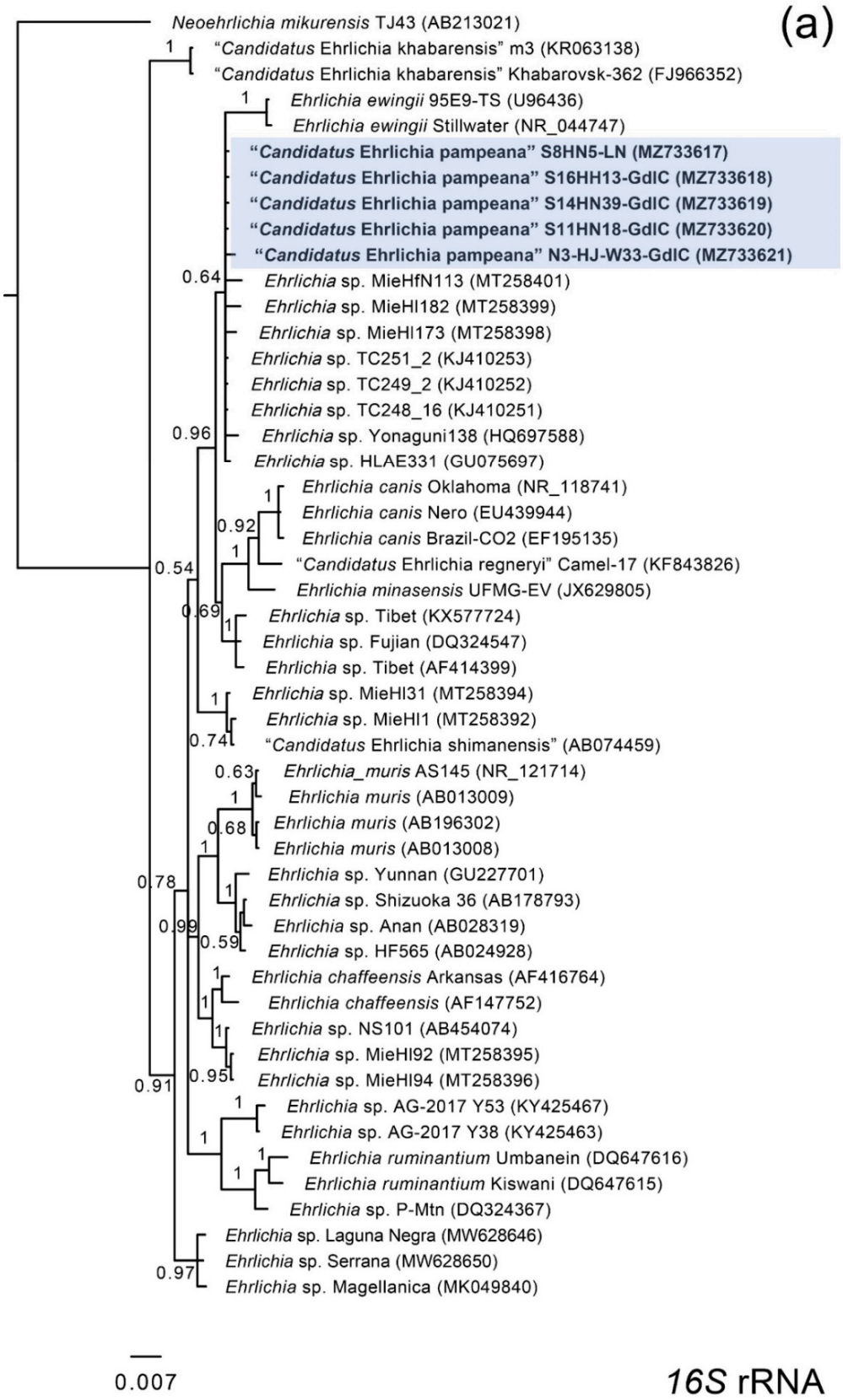

Figure 1. *Cont.*

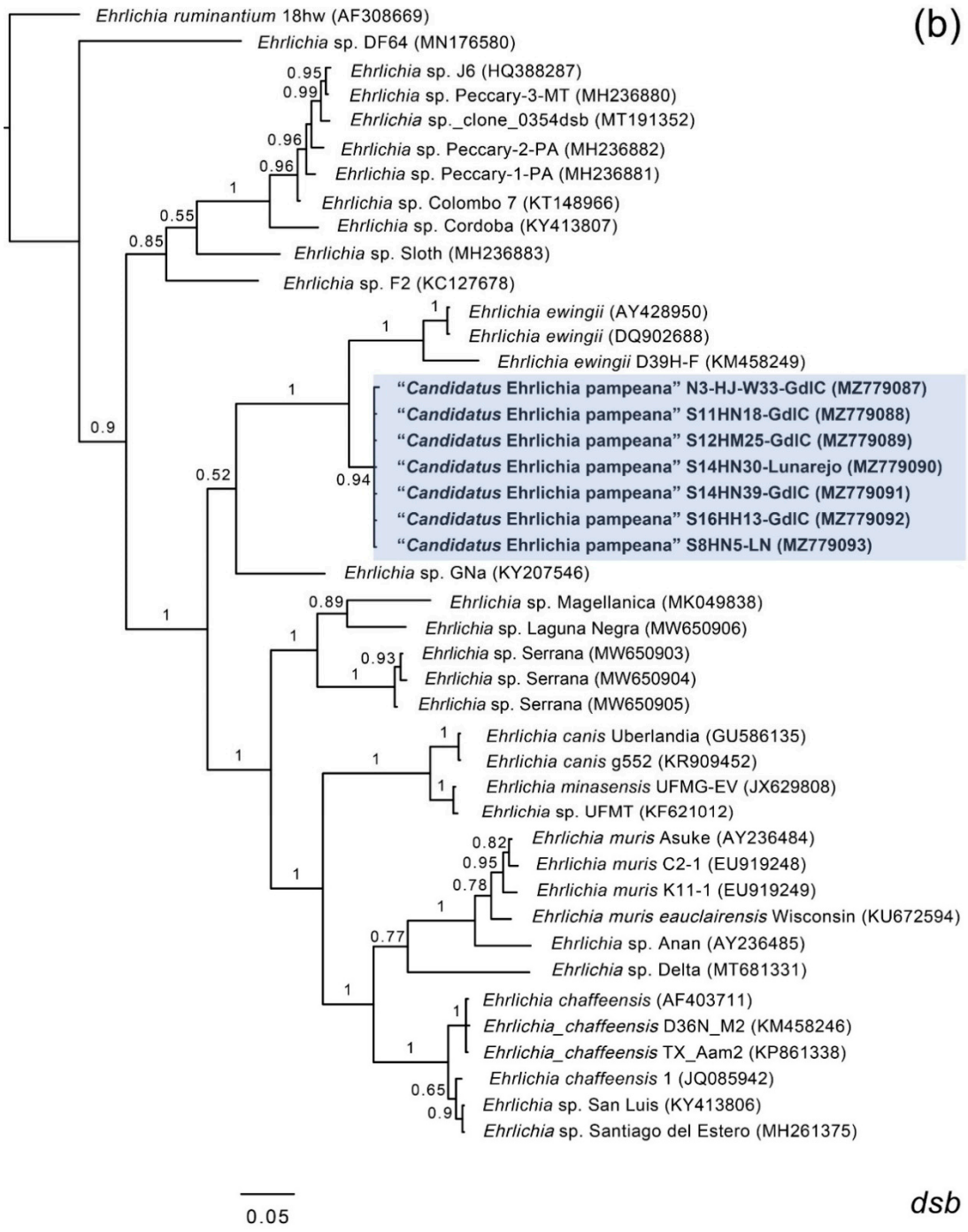

Figure 1. *Cont.*

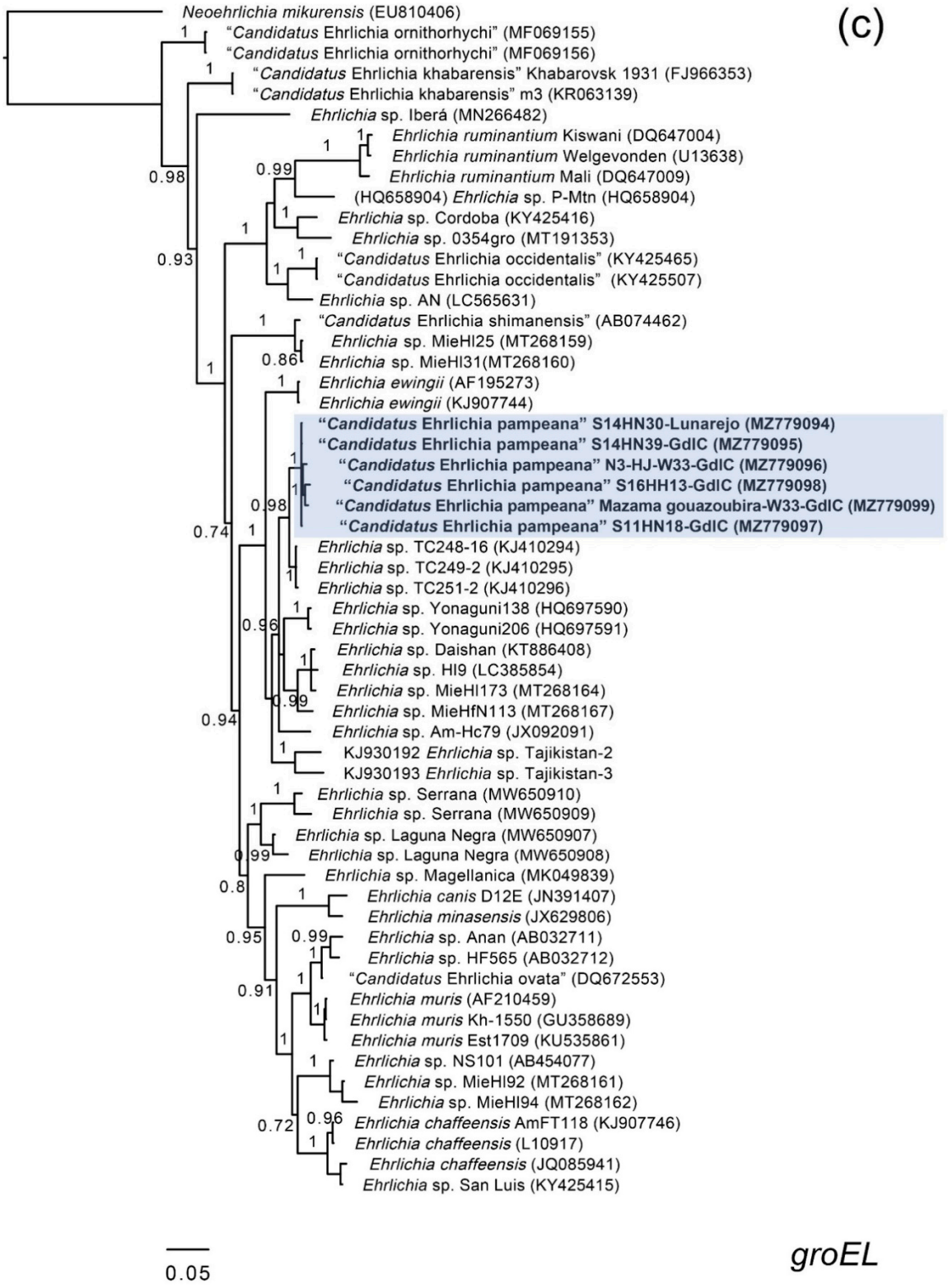

Figure 1. Bayesian phylogenetic analyses inferred for partial fragments of the genes (**a**) *16S* rRNA, (**b**) *dsb*, and (**c**) *groEL*. Bayesian posterior probabilities are indicated upon each branch. The positions of "*Candidatus* Ehrlichia pampeana" are highlighted with blue. Scale bar indicates the number of substitutions per nucleotide position. GenBank accession numbers are in brackets.

4. Discussion

In recent decades, molecular advances have favored the determination of novel species and strains of *Ehrlichia* in ticks from South America; for instance, *E. minasensis* and *E. canis* in *Rhipicephalus microplus* and *R. sanguineus,* respectively [32]. In addition, *Ehrlichia* cf. *chaffeensis*, and a series of strains (*Ehrlichia* sp. strain Córdoba, *Ehrlichia* sp. strain San Luis, *Ehrlichia* sp. strain Iberá, *Ehrlichia* sp. strain Jaguar, *Ehrlichia* sp. strain Delta, *Ehrlichia* sp. strain La Dormida, and a *Ehrlichia* sp.) were detected in ticks of the genus *Amblyomma* [13,33–39]. Recently, new *Ehrlichia* genotypes were described in *Ixodes uriae* from Chile and *Ixodes auritulus* from Uruguay [2,14]. Collectively, these findings suggest that different *Ehrlichia* species and genotypes are circulating in South American ecosystems.

The genetic distances and phylogenetic relationships for the *16S* rRNA, *groEL,* and *dsb* genes of the *Ehrlichia* sp. characterized in this study clearly denote the finding of a novel species related to the *Ehrlichia* species harbored by *Haemaphysalis* spp., *Hyalomma anatolicum,* and *D. nuttalli* ticks from Asia [3,40–45]. We propose its denomination as "*Candidatus* Ehrlichia pampeana". The species name is in allusion to the Pampa biome where positive ticks and deer were found. Remarkably, "*Ca.* E. pampeana" is also related to *E. ewingii* detected in *Amblyomma* spp. and dog blood from USA and *R. sanguineus* from Cameroon [46–50]. The topology of the phylogenetic trees also suggested that "*Ca.* E. pampeana" is closely related to *E. ewingii*. Although *16S* rRNA and *groEL* phylogenies indicated that other *Ehrlichia* spp. detected in ticks from Asia clustered with "*Ca.* E. pampeana", there are no *dsb* sequences available for the Asian Ehrlichiae genotypes, thus no phylogenetic relationship could be established with this locus (Figure 1b).

"*Candidatus* E. pampeana" is associated with the gray brocket deer since a fragment of the *groEL* gene was retrieved from the spleen of this cervid. This *Ehrlichia* sp. deer association was previously reported for two human-pathogenic *Ehrlichia* species such as *E. chaffeensis* and *E. ewingii* that use *Odocoileus virginianus* (white-tailed deer) as their main animal reservoir in the USA [9]. This fact, added to the detection of "*Ca.* E. pampeana" DNA in *H. juxtakochi* ticks, suggests that *M. gouazoubira* could act as a reservoir for this bacterium, which could be transmitted by its associated tick species (*H. juxtakochi).*

The molecular characterization of "*Ca.* E. pampeana" represents the third genotype of *Ehrlichia* determined in Uruguay, and the first report of an *Ehrlichia* sp. in *H. juxtakochi*, as well as for *Haemaphysalis* spp. from South America.

Human ehrlichiosis was documented in Uruguay more than ten years ago [15], and further cases have not been reported. Notably, *Amblyomma triste*, a tick that commonly bites humans in South America, has been positive to *Ehrlichia* spp. detection in Brazil and Argentina [37,51]; however, bacteria of this genus have not been detected in *A. triste* Uruguayan populations to date [37,52]. While the pathogenicity of "*Ca*. E. pampeana" for humans is uncertain, our results highlight eco-epidemiological features that might be relevant to suggest this novel *Ehrlichia* as a putative human pathogen as follows: (i) "*Ca*. E. pampeana" is phylogenetically closely related to *E. ewingii*, a recognized zoonotic pathogen [7], (ii) although *H. juxtakochi* parasitizes cervids, it has been also recorded feeding on humans [16,17], and (iii) the role of cervids as reservoirs for pathogenic *Ehrlichia* species has been previously suggested in North America [9].

Ehrlichiae are transstadially transmitted bacteria that need vertebrate hosts in order to thrive in nature [1]. For this reason, more studies are needed to determine the presence of "*Ca*. E. pampeana" in different *H. juxtakochi* hosts along the distribution range of this tick species. Moreover, further studies will be necessary to understand the eco-epidemiology of this novel bacteria and to assess its pathogenicity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9102165/s1, Table S1: Complete data of *Haemaphysalis juxtakochi* collected in vegetation for each site and detection of "*Candidatus* Ehrlichia pampeana".

Author Contributions: Conceptualization, M.L.F., S.M.-L., M.T.A.-F., and J.M.V.; data curation, M.L.F., and J.M.V.; methodology, M.L.F., S.M.-L., L.A.C., D.Q., S.R., M.T.A.-F., and J.M.V.; validation,

M.L.F., formal analysis, M.L.F., S.M.-L., M.T.A.-F., and J.M.V.; investigation, M.L.F., S.M.-L., L.A.C., D.Q., S.R., M.T.A.-F., and J.M.V.; resources, M.L.F., and J.M.V.; writing—original draft preparation, M.L.F., S.M.-L., M.T.A.-F., and J.M.V.; writing—review and editing, M.L.F., S.M.-L., L.A.C., D.Q., S.R., M.T.A.-F., and J.M.V.; validation, M.L.F.; supervision, J.M.V.; project administration, M.L.F. and J.M.V.; funding acquisition, M.L.F. and J.M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly supported by the Comisión Sectorial de Investigación Científica (Programa Iniciación a la Investigación 2017—Project ID 160) to M.L.F., and PEDECIBA (Programa de Desarrollo de las Ciencias Básicas)—Área Biología support 2020–2021 to J.M.V.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data presented in this study are available in the article.

Acknowledgments: We would like to thank Gustavo de Souza and Fernando Ramos (Colonia Don Bosco, Laguna Negra, Rocha); Eduardo Méndez, Alejandro Rodríguez, and Andrés de Mello (Reserva Natural Salus, Lavalleja); Hugo Pereda and Ricardo Palle (Gruta de los Cuervos, Tacuarembó); Daniel Casalás, Winderson Rodriguez da Cunha, and Lorena Ojeda (Amarillo, Rivera), for their collaboration during the field work.

Conflicts of Interest: The authors declare no conflict of interest.

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