SEBASTIÁN ALEJANDRO MUÑOZ LEAL

Study on *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* agents in ticks (Acari: Ixodoidea: Argasidae, Ixodidae) from Chile, and a taxonomic study on *Ornithodoros capensis* sensu lato (Acari: Argasidae) in South America

São Paulo

2017

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Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

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Orientador:

Prof. Dr. Marcelo Bahia Labruna

De acordo: _____

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Study on *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and Hepatozoon agents in ticks (Acari: Ixodoidea: Argasidae, Ixodidae) from Chile, and a taxonomic study on *Ornithodoros capensis sensu lato* (Acari: Argasidae) in South America / Sebastián Alejandro Muñoz Leal. -- 2017.
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CERTIFICADO

Certificamos que a proposta intitulada "Pesquisa de agentes dos gêneros *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* e *Hepatozoon* em carrapatos (Acari: Argasidae, Ixodidae) silvestres do Chile", protocolada sob o CEUA nº 9602030214, sob a responsabilidade de **Marcelo Bahia Labruna e equipe**; *Sebastián Muñoz Leal*; *Daniel Gonzalez Acuña* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 12/11/2014.

We certify that the proposal "título em inglês", utilizing 200 Non-Brazilian wild species (100 males and 100 females), protocol number CEUA 9602030214, under the responsibility of **Marcelo Bahia Labruna and team**; *Sebastián Muñoz Leal*; *Daniel Gonzalez Acuña* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 11/12/2014.

Finalidade da Proposta: [Pesquisa](#)

Vigência da Proposta: de [01/01/1](#) a [31/12/1](#)

Área: [Medicina Veterinária Preventiva E Saúde Animal](#)

Origem:	Não aplicável biotério		
Espécie:	Espécies silvestres não brasileiras	sexo:	Machos
Linhagem:	Roedores	idade:	a
		Peso:	a
Origem:	Não aplicável biotério		
Espécie:	Espécies silvestres não brasileiras	sexo:	Fêmeas
Linhagem:	Roedores	idade:	a
		Peso:	a

Registro IBAMA/Sisbio/Etc:

Método de Captura: [armadilhas tipo Sherman](#).

Resumo: A pesquisa de patógenos transmitidos por carrapatos no Chile é ainda incipiente. No presente projeto, carrapatos serão coletados em vida livre e de roedores silvestres em todas as regiões do Chile, e trazidos ao laboratório para pesquisa de microorganismos dos gêneros *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* e *Hepatozoon*, através de técnicas de biologia molecular. Roedores silvestres capturados em torno de 100 animais.

Local do experimento:

São Paulo, 18 de setembro de 2017

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

Claudia Madalena Cabrera Mori
Vice-Presidente
Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

EVALUATION FORM

Author: MUÑOZ LEAL, Sebastián Alejandro

Title: **Study on *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* agents in ticks (Acari: Ixodoidea: Argasidae, Ixodidae) from Chile, and a taxonomic study on *Ornithodoros capensis* sensu lato (Acari: Argasidae) in South America.**

Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date: ____/____/____

Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

DEDICATION

This work is dedicated to my parents, Ximena and René,
who early introduced me to nature
and encouraged me to keep studying it.

ACKNOWLEDGEMENTS

The current study represents the combined efforts of an international group of tick researchers and I am most grateful for the time that they have invested in order to contribute to this work.

In first place, I would like to thank Marcelo B. Labruna for the numerous advices provided until the last analyses in the course of this study. Without his expertise, this work would be likely to include many inaccurate statements. Long and fruitful talks on soft tick systematics with him guided a great part of this work. Extensive discussions on Argasidae were also shared with José M. Venzal, who in two occasions joined the fieldwork group, and actively participated in the expedition to Northern Chile and Southern Peru (2016), and to Northern Peru (2017).

The collection of the material and obtention of results in the initial phases of this work would never have been achieved without the strategic assistance of Daniel González-Acuña, who also enthusiastically contributed in fieldwork, and in the organization of the expeditions for the collection of material in several Islands in Chile (2015), and to the Southern region of Peru (2016). Much appreciated assistance was received from Thiago F. Martins, Nicolás Ferrada, Cecilia Aguayo, Iván Torres, Isabella Fontana, and Igor C. L. Acosta during fieldwork in Chile, and from Hermes Ribeiro Luz, Débora Panizzi Gallon, and Ádamo Gabriel Cavalcanti Limeira in Brazil. I wish to express my sincere thanks to them all for their efforts.

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A considerable contribution to the results of the current study was made by some of my laboratory fellows. I am deeply grateful to Francisco B. Costa, who taught me the PCR technique, and dedicated great efforts in cellular cultures to achieve *Rickettsia* isolations from Chilean ticks. Sincere thanks are also extended to Amália M. Barbieri, Fernanda Nieri and Yohana Acevedo, who also expended days in order to successfully isolate *Rickettsia* from soft ticks.

I wish to thank the technicians and assistant personnel from the laboratory for their indispensable task in preparing daily-work material, and in maintaining the cleanliness of the facilities.

Finally, I thank to the “Comisión Nacional de Investigación y Tecnología” (CONICYT, Chile) for the awarded grant (72140079).

RESUMO

MUÑOZ-LEAL, S. **Pesquisa dos agentes *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* e *Hepatozoon* em carrapatos (Acari: Ixodoidea: Argasidae, Ixodidae) do Chile e estudo taxonômico de *Ornithodoros capensis* sensu lato (Argasidae) na América do Sul** [Study on *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* agents in ticks (Acari: Ixodoidea: Argasidae, Ixodidae) from Chile, and a taxonomic study on *Ornithodoros capensis* sensu lato (Acari: Argasidae) in South America]. 2017. 200 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Até 2014, o conhecimento científico sobre a diversidade de Ixodoidea no Chile estava representado por 19 espécies e apenas agentes infecciosos dos gêneros *Borrelia* e *Rickettsia* haviam sido descritos. O objetivo deste estudo foi o de avaliar a ocorrência de outros patógenos transmitidos por carrapatos por meio de técnicas moleculares orientadas para a detecção de *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* e *Hepatozoon*. As sequências obtidas foram analisadas filogeneticamente, identificando-se suas posições em comparação à de organismos de papéis patogênicos já conhecidos. Como os agentes do gênero *Coxiella* apresentaram proximidade filogenética em relação a bactérias congêneras endossimbiontes, os dados sobre estas foram utilizados para realizar um estudo taxonômico em carrapatos do complexo *Ornithodoros capensis* sensu lato. Em geral, os resultados confirmam a presença de pelo menos três novas espécies de *Borrelia*, uma nova *Rickettsia*, e três novas espécies de *Hepatozoon* para a ciência. *Rickettsia amblyommatis*, *Rickettsia hoogstraalii* e *Rickettsia lusitaniae* foram inseridas como novos agentes associados a carrapatos no Chile. Embora alguns carrapatos fossem positivos para a presença de bactérias da família Anaplasmataceae, futuros estudos devem ser desenvolvidos para confirmar a sua condição específica, especialmente através da obtenção de maiores fragmentos do gene codificante para RNA 16S. Os organismos tipo *Coxiella* são específicos para cada uma das quatro espécies de carrapatos do grupo *O. capensis* analisados neste estudo. Portanto, constituem uma ferramenta de valor taxonômico para confirmar as identidades e limites genéticos destes. Finalmente, os resultados deste estudo adicionam pelo menos cinco novas espécies de carrapatos para a família Argasidae no Chile e apontam a ocorrência de vários morfotipos de condição incerta que precisam de maiores análises para esclarecer a com certeza a sua posição taxonômica.

Palavras-chave: Chile, carrapatos, doenças transmitidas por carrapatos, PCR, análise filogenético.

ABSTRACT

MUÑOZ-LEAL, S. **Study on *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* agents in ticks (Acari: Ixodoidea: Argasidae, Ixodidae) from Chile, and a taxonomic study on *Ornithodoros capensis* sensu lato (Acari: Argasidae) in South America.** [Pesquisa dos agentes *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* e *Hepatozoon* em carrapatos (Acari: Ixodoidea: Argasidae, Ixodidae) do Chile e estudo taxonômico de *Ornithodoros capensis* sensu lato (Argasidae) na América do Sul]. 2017. 200 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Until 2014, scientific knowledge on the diversity of Chilean Ixodoidea summarized 19 species and only agents of *Borrelia* and *Rickettsia* genera had been detected. The objectives of this study were to evaluate the occurrence of further agents of *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* by means of molecular tools. Obtained sequences were inserted into a phylogenetic context in order to evaluate their relatedness to microorganisms of known pathogenic roles. As agents of *Coxiella* genus resulted to be related to endosymbiotic bacteria, data on these organisms was used to perform a taxonomic study with ticks of the *Ornithodoros capensis* sensu lato complex. The results confirm that Chilean ticks harbor at least three new borrelial, one new rickettsial, and three new *Hepatozoon* species for science. Moreover, *Rickettsia amblyommatis*, *Rickettsia hoogstraalii* and *Rickettsia lusitaniae* are added to the list of Chilean rickettsiae. Although ticks were positive to Anaplasmataceae PCRs, an accurate study including longer fragments of the 16S RNA targeted gene must be performed in order to confirm their specific identity. *Coxiella*-like endosymbionts are specific of every of the four *O. capensis* s. l. species analyzed in this study, and therefore constitute a useful tool in order to confirm the identities and define genetic boundaries of ticks of this group in South America. Finally, the results of this study add at least five new species of Argasidae family into Chilean fauna of ticks, and point the occurrence of several forms that need further assessment in order to accurately confirm their identities.

Keywords: Chile, ticks, tick-borne microorganisms, PCR, phylogenetic analysis

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1. INTRODUCTION

South American fauna of ticks merge life cycles with different orders of amphibian, reptile, birds (GUGLIELMONE et al., 2003; OGRZEWALSKA; PINTER, 2016), and micro and macromammals (GUGLIELMONE et al., 2003; DURDEN, 2006; LUZ et al., 2016; MUÑOZ-LEAL et al., 2016; SPONCHIADO et al., 2015), including humans (GUGLIELMONE et al., 2006). Interest in tick-borne diseases from a human and domestic animal health point of view has promoted the realization of deep research on tick-borne microorganisms from this region. Culminating results have demonstrated that some native species are true or putative vectors that transmit pathogenic bacteria (LABRUNA, 2009; SILVEIRA et al., 2012; PACHECO et al., 2013; IVANOVA et al., 2014) and protozoan species (FORLANO et al., 2005; SOARES et al., 2014, 2015)

In a parallel way, research on South American ticks has also extended its scope to the wild interface where these parasites coexist with their vectored agents. Directly related, research on taxonomy has also contributed to the knowledge of this group of parasites. Fruitful ongoing research on tick taxonomy and tick-borne microorganisms comes from Brazil (FORLANO et al., 2005; BARROS-BATTESTI et al., 2006; LABRUNA, 2009; LABRUNA et al., 2016; MARTINS et al., 2016; COSTA et al., 2017; MUÑOZ-LEAL et al., 2017), Argentina, Uruguay, and Chile (GUGLIELMONE et al., 2003; NAVA et al., 2017).

The current study started in early 2014, when the knowledge on Chilean diversity of ticks included seven species in the Argasidae and 12 in the Ixodidae

families, and only bacteria of *Rickettsia* genus (ABARCA et al., 2012, 2013), and one species of *Borrelia* (IVANOVA et al., 2014) were known to be harbored by native ticks. Considering this scenario, this study aimed to extend this knowledge exploring the diversity of bacteria of genus *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, and *Rickettsia*, and of protozoans of *Hepatozoon* genus in Chilean ticks. Based in preliminary results, and attempting to insert the findings on these microorganisms into a taxonomical context, the following two hypotheses were posed: 1) native ticks from Chile are potential vectors of *Anaplasma*, *Borrelia*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* species; and 2) the representatives of the *Ornithodoros capensis* sensu lato group from South America harbor specific *Coxiella*-like microorganisms, which genetic sequences can be used as taxonomic tools to assist specific diagnosis of their associated ticks.

In order to address both questions, the objectives were set as follows:

- 1) To identify the analyzed ticks by a morphological and molecular approach.
- 2) To describe new species of ticks.
- 3) To detect and molecularly characterize microorganisms of *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Rickettsia* and *Hepatozoon* in native ticks from Chile.
- 4) To insert the genetically characterized microorganisms into a phylogenetic context, assessing their relatedness with tick-borne agents of known pathogenicity.

Along the development of this study, the collection of ticks implied the capture of their associated vertebrate hosts, and also to carry active searches in the field in order to find them within burrows, nests and caves. In total, this study comprised 14 collection localities in Chile. In addition, eight localities along the Peruvian seaboard were also prospected in order to enlarge the sampling area for the study concerning to *O. capensis* sensu lato (s. l.). Maps with the geographic location of each study site are presented in each chapter.

In Chile, ecosystems are shaped by a desert regime in the northern regions, a Mediterranean climate in the middle, and a cold Oceanic influence towards the south (DI CASTRI, 1968; DI CASTRI; HAJEK, 1976). The eastern border of the country limits with the Andes Mountains, and the western border with the Pacific Ocean. With

the exception of amphibians, in this country all classes of terrestrial vertebrates have been reported as hosts for Ixodoidea. Major native terrestrial mammals inhabiting inland Chilean territories belong to the Artiodactyla (seven species) and Carnivora orders (15 species); small mammals are represented by four species of marsupials of the orders Didelphimorphia, Microbiotheria, and Paucituberculata; the order Xenarthra is represented by three species, 68 species compose the order Rodentia, and 11 the order Quiroptera (IRIARTE, 2008). Five hundred and thirty one species of birds inhabit marine and terrestrial ecosystems along the country (COUVE; VIDAL; RUIZ T., 2016), and the herpetofauna is composed by 134 species of inland reptiles (DEMANGEL, 2016), and 62 of amphibians (CORREA; DONOSO; ORTIZ, 2016).

The results of this study have been adapted for publication into five separated manuscripts. The two first chapters present the descriptions of new species of Chilean argasids; chapters three and four deal with detected microorganisms, and the final chapter concerns to the findings on *O. capensis* s. l. as follows:

- a) A new species of *Ornithodoros* (Acari: Argasidae) from desert areas of northern Chile (Ticks Tick Borne Dis. 2016; 7[5]: 901-910. doi: 10.1016/j.ttbdis.2016.04.008)
- b) Description of two new species of soft ticks (Acari: Argasidae: *Ornithodoros*) from Central Chile. (To be published).
- c) Bacteria of the genus *Rickettsia* associated to soft ticks (Acari: Argasidae) of Northern and Central Chile. (To be published)
- d) Anaplasmataceae, *Borrelia*, and *Hepatozoon* agents associated to native ticks (Acari: Argasidae, Ixodidae) from Chile. (To be published)
- e) The *Ornithodoros capensis* group (Acari: Argasidae) of the Neotropical region: genetic identity, morphology, and geographical distribution of four species in South America. (To be published)

1.1. REFERENCES

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2. A NEW SPECIES OF *Ornithodoros* (ACARI: ARGASIDAE) FROM DESERT AREAS OF NORTHERN CHILE

2.1. INTRODUCTION

The worldwide specific richness of soft ticks (Argasidae) is currently represented by *ca.* 200 species (Vial and Camicas 2009, Guglielmone et al. 2010, Nava et al. 2010, 2013, Dantas-Torres et al. 2012, Heath 2012, Venzal et al. 2012, 2013, 2015, Trape et al. 2013, Barros-Battesti et al. 2015). Combining the use of morphologically significant characters and molecular markers, 15 new species of soft ticks have been described in the last 12 years for the Neotropical Zoogeographic Region (Barros-Battesti et al. 2015, Venzal et al. 2015). This fact strongly suggests that the number of species of soft ticks is underestimated in the Neotropics.

Ticks of genus *Ornithodoros* Koch are nidicolous and cave dweller parasites well adapted to survive in arid environments (Hoogstraal 1985, Oliver 1989). The northern half of Chile (roughly 18° - 33°S) is characterized by large extensions of arid ecosystems, including within its range the Atacama Desert, one of the driest regions of the world (Clarke 2006). To date, the Chilean diversity of soft ticks is represented by *Otobius megnini* (Dugès 1884) (González-Acuña and Guglielmone 2005), two species of *Argas* and six species of *Ornithodoros*, being five of them described for the northern arid regions of the country (González-Acuña and Guglielmone 2005, González-acuña et al. 2008, Venzal et al. 2013, 2015). Based on larval and adult morphology, and on phylogenetic analyses of the 16S rRNA mitochondrial gene, here we present the description of a new species of *Ornithodoros* associated with reptiles in desert areas of northern Chile.

2.2. MATERIAL AND METHODS

2.2.1. Ticks

Ticks were collected from the environment and from lizards in three localities from northern Chile (Fig. 1). Reptiles were captured according to the licenses given by the Servicio Agrícola and Ganadero (SAG) and the Corporación Nacional Forestal (CONAF) (documents 6007/2014 and 033/2014 respectively). Free-living adults (10 females and 17 males) belonging to the genus *Ornithodoros* were collected from rock crevices in Pan de Azúcar National Park (PANP, 26°17'S; 70°38'W; 2014), and a total of 139 larvae of *Ornithodoros* were collected on *Liolaemus bisignatus* (Philippi 1860) (Squamata: Liolaemidae) in PANP (n = 53) and Llanos de Challe National Park (n = 86) (LCNP, 28°11'S; 71°09'W; 2011-2014), both sites located in the Atacama region. Also, two *Ornithodoros* larvae were found on *Callopistes maculatus* Gravenhorst 1838 (Squamata: Teiidae) in Las Chinchillas National Reserve (LCNR, 31°30'S; 71°06'W; 2011-2014), Coquimbo region. Larvae from reptiles were collected in 70 - 90% ethanol and adult ticks were brought alive to the laboratory and placed in incubators at 25°C and 80% of relative humidity. Under these conditions, two females laid eggs and we obtained the unfed larvae. Ten laboratory-reared and 39 field-collected larvae were clarified in 20% KOH solution, mounted in semi-permanent slides for microscopy using Hoyer's medium, and measured using a Nikon Eclipse E200 optical microscope. All measurements are given in millimeters (mm), with the mean followed by the standard deviation and range in parentheses. Terminology for larval chaetotaxy and measurements followed Venzal et al. (2008, 2013).

Slightly engorged females, males and unengorged larvae were prepared according to the methods presented by Corwin et al. (1979) and used to obtain scanning electron microscopy images. The micrographs were obtained using a JEOL JMS-5900 scanning electron microscope (JEOL, Tokyo, Japan). Terminology for the description of adult characters followed Cooley and Kohls (1944) and (Nava et al. 2013). One of the females from which unfed larvae were obtained in the laboratory,

one field-collected larva from PANP and one larva from PNLC were submitted to DNA extraction using the Guanidine Isothiocyanate technique (Sangioni et al. 2005).

Figure 1. Map of Chile showing the localities where *Ornithodoros atacamensis* n. sp. specimens were collected.

A principal component analysis (PCA) based on Pearson correlation matrix was performed for 41 morphometric variables (Table 1) of unengorged larvae in order to discriminate the relationship between phenotypically closely species. To this effect, measurements of *Ornithodoros guaporensis* Nava, Venzal and Labruna 2013, *Ornithodoros hasei* (Schulze, 1935), *Ornithodoros puertoricensis* Fox 1947, *Ornithodoros rioplatensis* Venzal, Estrada-Peña and Mangold 2008, and *Ornithodoros talaje* sensu stricto (s. s.) (Guérin-Méneville, 1849) were included.

In order to obtain unengorged adult specimens for allowing a better visualisation of morphological characters, field-collected *Ornithodoros* adults were maintained unfed in the incubator for 14 months. Thereafter, females (n = 2) were examined using a Zeiss Stemi SV11 stereomicroscope, and their external morphology was compared with other morphologically related Neotropical

Ornithodorinae. To this effect, we analysed the following unengorged females deposited in the tick collection “Coleção Nacional de Carrapatos” (CNC) of the Faculty of Veterinary Medicine of the University of São Paulo, Brazil: *O. guaporensis* from Bolivia (n = 2; CNC-2305); *O. rioplatensis* from Uruguay (n = 2; CNC-3254), and *O. puertoricensis* from Panama (n = 4; CNC-3255). Photographs of examined females were taken with a Canon PowerShot SX700HS camera. We only analysed females because the external morphology of adult argasids is very similar for both sexes (with the exception of the genital area).

2.2.2. Phylogenetic analysis

Obtained sense and antisense sequences of the mitochondrial 16S rRNA gene were assembled, manually edited, and an alignment with other sequences of Neotropical congeneric species available in GenBank. Alignment was performed using the Clustal W program (Thompson et al. 1994), and reconnection branch swapping with missing data; all positions were equally weighed. A Bayesian analysis was performed using MrBayes v3.1.2 with 1,000,000 generations and trees being sampled every 1,000 generations, running 4 times beginning with random starting trees. The General Time Reversible model (GTR) was used combined with the models of gamma distribution (G) (Huelsenbeck and Ronquist 2001). The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability (BPP). Sequences of *Ixodes holocyclus* Neumann 1899 (AB051845) and *Ixodes uriae* White 1852 (AB030017) were used as out-groups.

Table 1. Average and range (mm) of measurements from *O. atacamensis*, *O. puertoricensis*, *O. rioplatensis*, *O. guaporensis*, *O. hasei*, and *O. talaje* used for de PCA. The number of measured ticks is indicated in parenthesis.

	<i>O. atacamensis</i> (10)	<i>O. puertoricensis</i> (10)	<i>O. rioplatensis</i> (10)	<i>O. guaporensis</i> (10)	<i>O. hasei</i> (5)	<i>O. talaje</i> (5)
Dorsal plate: length	0.302 (0.238 - 0.318)	0.219 (0.202 - 0.236)	0.262 (0.242 - 0.276)	0.290 (0.271 - 0.305)	0.255 (0.191 - 0.296)	0.251 (0.245 - 0.262)
Dorsal plate: width	0.202 (0.194 - 0.217)	0.172 (0.163 - 0.183)	0.177 (0.166 - 0.187)	0.220 (0.212 - 0.232)	0.152 (0.142 - 0.171)	0.177 (0.170 - 0.185)
Dorsal setae (pairs): total	17	18	20	20	19	17
Dorsal setae (pairs): dorsolateral	12	14	16	16	15	13
Dorsal setae (pairs): central	5	4	4	4	4	4
Central setae: C ₁ length	0.067 (0.063 - 0.073)	0.094 (0.073 - 0.105)	0.094 (0.085 - 0.100)	0.094 (0.083 - 0.100)	0.083 (0.073 - 0.086)	0.083 (0.078 - 0.085)
Central setae: C ₂	0.058 (0.051 - 0.064)	0.086 (0.073 - 0.093)	0.081 (0.073 - 0.090)	0.088 (0.085 - 0.093)	0.069 (0.069 - 0.071)	0.075 (0.073 - 0.078)
Central setae: C ₃	0.060 (0.055 - 0.065)	0.085 (0.068 - 0.095)	0.078 (0.073 - 0.085)	0.082 (0.075 - 0.090)	0.070 (0.064 - 0.078)	0.071 (0.066 - 0.078)
Central setae: C ₄	0.058 (0.053 - 0.063)	0.086 (0.071 - 0.095)	0.079 (0.063 - 0.090)	0.092 (0.088 - 0.097)	0.075 (0.069 - 0.086)	0.068 (0.063 - 0.073)
Sternal setae: St ₁	0.046 (0.040 - 0.051)	0.057 (0.049 - 0.063)	0.064 (0.061 - 0.066)	0.069 (0.066 - 0.073)	0.060 (0.054 - 0.069)	0.055 (0.049 - 0.061)
Sternal setae: St ₂	0.043 (0.039 - 0.050)	0.059 (0.049 - 0.071)	0.061 (0.052 - 0.068)	0.055 (0.049 - 0.061)	0.056 (0.054 - 0.059)	0.060 (0.053 - 0.063)
Sternal setae: St ₃	0.044 (0.041 - 0.046)	0.056 (0.044 - 0.063)	0.063 (0.056 - 0.068)	0.059 (0.058 - 0.061)	0.057 (0.054 - 0.061)	0.053 (0.053 - 0.056)
Circumanal setae: Ca ₁	0.041 (0.033 - 0.050)	0.051 (0.044 - 0.058)	0.054 (0.045 - 0.061)	0.053 (0.044 - 0.061)	0.054 (0.049 - 0.059)	0.047 (0.037 - 0.053)
Circumanal setae: Ca ₂	0.047 (0.041 - 0.052)	0.068 (0.056 - 0.078)	0.067 (0.060 - 0.075)	0.071 (0.066 - 0.073)	0.066 (0.064 - 0.071)	0.055 (0.050 - 0.057)
Circumanal setae: Ca ₃	0.057 (0.050 - 0.064)	0.094 (0.078 - 0.105)	0.089 (0.078 - 0.097)	0.102 (0.097 - 0.107)	0.078 (0.073 - 0.081)	0.070 (0.067 - 0.073)
Postcoxal setae: Pc	0.031 (0.028 - 0.033)	0.046 (0.039 - 0.053)	0.051 (0.046 - 0.058)	0.040 (0.034 - 0.046)	0.059 (0.049 - 0.069)	0.038 (0.036 - 0.041)
PMS	0.040 (0.025 - 0.048)	0.067 (0.049 - 0.078)	0.057 (0.052 - 0.063)	0.074 (0.068 - 0.080)	0.055 (0.049 - 0.061)	0.053 (0.049 - 0.056)
Length of basis capituli (a)	0.125 - (0.105 - 0.140)	0.116 (0.098 - 0.129)	0.121 (0.112 - 0.134)	0.133 (0.124 - 0.139)	0.141 (0.133 - 0.147)	0.133 (0.127 - 0.137)
Length of basis capituli (b)	0.152 (0.130 - 0.169)	0.128 (0.110 - 0.149)	0.136 (0.117 - 0.164)	0.156 (0.146 - 0.166)	0.182 (0.176 - 0.186)	0.154 (0.142 - 0.160)
Length of basis capituli (c)	0.342 (0.315 - 0.365)	0.367 (0.323 - 0.392)	0.370 (0.350 - 0.392)	0.441 (0.431 - 451)	0.362 (0.346 - 0.376)	0.326 (0.320 - 0.330)
Width of basis capituli	0.202 (0.180 - 0.220)	0.156 (0.146 - 0.171)	0.161 (0.150 - 0.171)	0.180 (0.173 - 0.193)	0.236 (0.228 - 0.240)	0.194 (0.185 - 0.205)
Palpal length	0.244 (0.236 - 0.254)	0.282 (0.263 - 0.293)	0.254 (0.250 - 0.262)	0.323 (0.310 - 0.332)	0.252 (0.240 - 0.282)	0.220 (0.215 - 0.227)
Length article I	0.005 (0.052 - 0.061)	0.052 (0.049 - 0.054)	0.054 (0.049 - 0.062)	0.063 (0.056 - 0.083)	0.063 (0.054 - 0.069)	0.052 (0.050 - 0.057)
Length article II	0.086 (0.082 - 0.092)	0.108 (0.100 - 0.112)	0.089 (0.080 - 0.097)	0.129 (0.122 - 0.134)	0.080 (0.073 - 0.086)	0.069 (0.062 - 0.075)
Length article III	0.086 (0.082 - 0.090)	0.094 (0.085 - 0.100)	0.084 (0.075 - 0.090)	0.106 (0.085 - 0.114)	0.067 (0.061 - 0.073)	0.075 (0.072 - 0.077)
Length article IV	0.037 (0.035 - 0.039)	0.037 (0.034 - 0.041)	0.036 (0.032 - 0.040)	0.035 (0.029 - 0.039)	0.046 (0.044 - 0.049)	0.030 (0.030 - 0.032)

Table 1. (Continued).

	<i>O. atacamensis</i> (10)	<i>O. puertoricensis</i> (10)	<i>O. rioplatensis</i> (10)	<i>O. guaporensis</i> (10)	<i>O. hasei</i> (5)	<i>O. talaje</i> (5)
Width article I	0.025 (0.024 - 0.027)	0.025 (0.024 - 0.027)	0.026 (0.025 - 0.027)	0.027 (0.024 - 0.032)	0.032 (0.029 - 0.037)	0.031 (0.030 - 0.032)
Width article II	0.035 (0.034 - 0.037)	0.033 (0.032 - 0.034)	0.036 (0.035 - 0.037)	0.032 (0.024 - 0.036)	0.036 (0.034 - 0.037)	0.037 (0.035 - 0.037)
Width article III	0.035 (0.034 - 0.037)	0.033 (0.032 - 0.034)	0.032 (0.030 - 0.032)	0.033 (0.032 - 0.034)	0.034 (0.029 - 0.039)	0.033 (0.030 - 0.035)
Width article IV	0.022 (0.022 - 0.022)	0.020 (0.019 - 0.022)	0.021 (0.020 - 0.022)	0.020 (0.019 - 0.024)	0.023 (0.022 - 0.024)	0.018 (0.017 - 0.020)
Hypostome: length (d)	0.219 (0.205 - 0.230)	0.252 (0.232 - 0.266)	0.243 (0.237 - 0.250)	0.309 (0.305 - 0.315)	0.212 (0.203 - 0.218)	0.197 (0.187 - 0.200)
Hypostome: length (e)	0.182 (0.178 - 0.190)	0.255 (0.207 - 0.251)	0.231 (0.255 - 0.237)	0.277 (0.268 - 0.285)	0.176 (0.174 - 0.179)	0.169 (0.162 - 0.175)
Hypostome: width base	0.049 (0.046 - 0.051)	0.052 (0.049 - 0.054)	0.054 (0.050 - 0.057)	0.047 (0.044 - 0.049)	0.061 (0.059 - 0.064)	0.053 (0.045 - 0.055)
Apical dental formula	3	3	3	3	3	3
Median dental formula	2	3	3	2	3	2
Basal dental formula	2	2	2	2	2	2
Denticles in hypostomal row 1	21 (19 - 22)	27	24 (23 - 25)	26 (26 - 27)	18	18 (17 - 19)
Denticles in hypostomal row 2	20 (18 - 22)	25	22 (21 - 24)	25 (25 - 26)	18 (17 - 18)	17 (16 - 17)
Denticles in hypostomal row 3	11 (10 - 12)	15	12 (11 - 13)	12 (11 - 13)	12 (11 - 12)	10 (9 - 11)
Tarsus I: length	0.171 (0.165 - 0.176)	0.176 (0.171 - 0.180)	0.176 (0.171 - 0.183)	0.174 (0.168 - 0.180)	0.175 (0.171 - 0.184)	0.182 (0.171 - 0.190)
Tarsus I: width	0.057 (0.053 - 0.063)	0.059 (0.054 - 0.061)	0.058 (0.056 - 0.061)	0.058 (0.056 - 0.058)	0.063 (0.059 - 0.069)	0.060 (0.058 - 0.063)

(a) Length of basis capituli to Ph1

(b) Length of basis capituli to insertion of hypostome

(c) Length of basis capituli to tip hypostome

(d) Measured to point of Ph1

(e) Measured to point of insertion of hypostome in basis capituli

2.3. RESULTS

2.3.1. Descriptions

Ixodida Leach, 1815

Argasidae Canestrini, 1890

Ornithodoros Koch, 1844

Ornithodoros atacamensis n. sp. Muñoz-Leal, Venzal & González-Acuña

Larva (Figs. 2 - 3)

Body: Idiosoma subcircular. Length including capitulum: 0.801 ± 0.028 (0.761-0.834), and not including capitulum: 0.474 ± 0.020 (0.438-0.499), width: 0.489 ± 0.035 (0.435-0.536). Dorsum: Dorsal plate pyriform, length 0.302 ± 0.010 (0.283-0.318), width: 0.202 ± 0.007 (0.194-0.217). Dorsal surface provided with 17 pairs of setae, 7 anterolateral, 5 central and 5 posterolateral. Anterolateral setae (Al): Al₁ length 0.067 ± 0.003 (0.063-0.074), Al₂ length 0.065 ± 0.004 (0.056-0.071), Al₃ length 0.061 ± 0.005 (0.052-0.067), Al₄ length 0.063 ± 0.003 (0.059-0.066), Al₅ length 0.063 ± 0.003 (0.059-0.067), Al₆ length 0.063 ± 0.003 (0.056-0.066), Al₇ length 0.066 ± 0.003 (0.060-0.071). Central setae (C): C₁ length 0.067 ± 0.003 (0.063-0.073), C₂ length 0.058 ± 0.004 (0.051-0.064), C₃ length 0.060 ± 0.003 (0.055-0.065), C₄ length 0.058 ± 0.003 (0.053-0.063), C₅ length 0.056 ± 0.005 (0.049-0.064). Posterolateral setae (Pl): Pl₁ length 0.058 ± 0.004 (0.053-0.067), Pl₂ length 0.063 ± 0.003 (0.059-0.066), Pl₃ length 0.059 ± 0.003 (0.053-0.062), Pl₄ length 0.059 ± 0.004 (0.052-0.066), Pl₅ length 0.058 ± 0.002 (0.054-0.061).

Venter: Ventral surface provided with 7 pairs of setae plus 1 pair on anal valves, posteromedian seta present. Three pairs of sternal setae (St): St₁ length 0.046 ± 0.004 (0.040-0.051), St₂ length 0.043 ± 0.003 (0.039-0.050), St₃ length 0.044 ± 0.002 (0.041-0.046); three pairs of circumanal setae (Ca): Ca₁ length 0.041 ± 0.005 (0.033-0.050), Ca₂ length 0.047 ± 0.004 (0.041-0.052), Ca₃ length 0.057 ± 0.004

(0.050-0.060); posteromedian setae (Pm) length 0.040 ± 0.007 (0.025-0.048), postcoxal setae (Pc) length 0.031 ± 0.002 (0.028-0.033).

Capitulum: Basis capituli pentagonal, posterior margin slightly curved. Length from posterior margin of basis capituli to posthypostomal setae Ph_1 0.126 ± 0.011 (0.105-0.140), length from posterior margin of basis capituli to insertion of hypostome 0.153 ± 0.014 (0.130-0.169), length from posterior margin of basis capituli to apex of hypostome 0.342 ± 0.015 (0.315-0.365), width 0.202 ± 0.013 (0.180-0.220). Two pairs of posthypostomal setae; Ph_1 length 0.012 ± 0.001 (0.011-0.012), Ph_2 length 0.031 ± 0.003 (0.024-0.034), distance between Ph_1 setae 0.027 ± 0.003 (0.023-0.031), distance between Ph_2 setae 0.067 ± 0.004 (0.060-0.075). Palpi total length 0.244 ± 0.006 (0.236-0.254), segmental length/width from I-IV: (I) 0.055 ± 0.003 (0.052-0.061) / 0.025 ± 0.001 (0.024-0.027), (II) 0.086 ± 0.003 (0.082-0.090) / 0.035 ± 0.001 (0.034-0.037), (III) 0.086 ± 0.003 (0.082-0.090) / 0.035 ± 0.001 (0.034-0.037), (IV) 0.038 ± 0.001 (0.035-0.039) / 0.022 ± 0.001 (0.021-0.022). Setae number on palpal articles I-IV: (I) 0, (II) 4, (III) 5, (IV) 9. Hypostome: length from Ph_1 to apex 0.219 ± 0.008 (0.205-0.230), length from insertion of hypostome in basis capituli to apex 0.192 ± 0.004 (0.188-0.198), length of hypostome from apex to inferior toothed portion 0.185 ± 0.003 (0.178-0.190), width in medial basis portion of hypostome 0.049 ± 0.002 (0.046-0.051), width in basis portion of hypostome 0.049 ± 0.001 (0.046-0.051); arises from the basis on a median extension, pointed apically. Dentition formula 3/3 in anterior third, 2/2 towards the base, file 1 with 19 to 22 denticles (typically 21), file 2 with 18 to 21 (typically 20) and file 3 with 10 to 12 denticles (typically 11).

Legs: Tarsus I length 0.171 ± 0.005 (0.165-0.176), tarsus I width 0.057 ± 0.004 (0.053-0.063). Setal formula of tarsus I: 1 pair apical (A), 1 distomedian (DM), 5 paracapsular (PC), 1 posteromedian (PM), 1 pair basal (B), 1 pair apicoventral (AV), 1 pair midventral (MV), 1 pair basiventral (BV), and 1 pair posterolateral (PL). Capsule of Haller's organ: rounded and without reticulations. Coxa I, II, and III with one ventral spur projecting towards the trochanter. Each coxa provided with two ventral setae.

Figure 2. *Ornithodoros atacamensis* n. sp. drawing of larva. (A) Dorsal. (B) Ventral. (C) Tarsus I. Scale in millimeters.

Figure 3. *Ornithodoros atacamensis* n. sp. scanning electron microscopy of the larva. (A) Dorsal idiosoma. (B) Basis capitulum and hypostome.

Female (Fig. 4)

Body: Body flat and oval, 5.9 ± 0.6 (5.3–6.7) long, 3.5 ± 0.3 (3.2–3.9) wide. Posterior margin rounded, lateral margins gently curved along the idiosoma and anteriorly converging to a slightly pointed apex. Dorsum: integument covered by a cell-like configuration of mammillae, slightly larger in marginal regions than in the center of the body. Some mammillae provided of a little seta. Middle of the idiosoma with numerous discs below the level of mammillae arranged in groups, not present at the margins. Lateral suture absent. Venter: Mammillae covering the ventral surface with few discs only present in postcoxal grooves. Genital area located between posterior ends of coxae I, anterior labia larger than posterior. Anus located posteriorly to coxae IV and surrounded with a smooth mammillated anal ring; preanal groove present; median mammillated depression reaching the postanal groove. Spiracular plate small and above coxa IV.

Capitulum: Capitulum situated ventrally to the hood in a well-defined camerostome. Cheeks movable, large and elongate, covering palps, hypostome and mouth parts. Basis capituli covered by irregular transverse wrinkles and micro-mammillated, 0.180 ± 0.015 (0.155–0.196) long, 0.232 ± 0.010 (0.218–0.243) wide, with one pair of long posthypostomal setae, one pair of postpalpal setae approximately third of the size of the posthypostomal setae. Palps free, article I micro-mammillated and with a smooth knife-edge flange in its median side. Hypostome rounded apically, with dental formula 2/2; small crenulations apically, anterior portion with two denticles in each row and then smaller denticles toward the base. Hood small.

Legs: long, entirely micro-mammillated with exception for coxae I–IV, which are partially mammillated (coxa IV almost unmammillated). Coxae I and II separated by a mammillated integument portion; coxae II to IV contiguous. Tarsi narrow without humps and protuberances, provided with stout bifid claws. Pulvilli absent.

Male (Fig. 5)

Similar to the female except in the genital area. Body 5.0 ± 0.2 (4.6–5.4) long, 3.0 ± 0.2 (2.8–3.3) wide. Basis capituli 0.177 ± 0.012 (0.162–0.204) long, 0.215 ± 0.008 (0.205–0.235) wide.

Figure 4. *Ornithodoros atacamensis* n. sp. scanning electron microscopy of the female. (A) Dorsal idiosoma. (B) Ventral idiosoma. (C) Ventral capitulum. (D) Genital opening.

Figure 5. *Ornithodoros atacamensis* n. sp., scanning electron microscopy of the male. (A) Dorsal idiosoma. (B) Ventral idiosoma. (C) Ventral capitulum. (D) Genital opening.

Type host: *Liolaemus bisignatus* Philippi 1860 (Squamata: Liolaemidae).

Type locality: Pan de Azúcar National Park (26°17'S; 70°38'W), Atacama Region, Chile.

Type specimens: Holotype larva mounted in a slide, allotype larva and two paratype larvae mounted in separated slides, host: *Liolaemus bisignatus*, Pan de Azúcar National Park, Atacama Region, Chile, coll. S. Muñoz-Leal and D. González-Acuña, December 8, 2014, deposited in USNTC, Georgia Southern University, Statesboro, USA (USNMENT 00862222, 00862223, 00862890, 00862891, respectively).

Paratypes: Ten larvae in 70 % ethanol, one female and one male, same locality, collectors and data of the holotype, deposited in USNTC (USNMENT 00862892, 00862893). Ten larvae in 70% ethanol, same host, locality, collectors and data for the holotype deposited in INTA, Rafaela, Argentina (INTA 2306). Ten larvae in 70% ethanol, same host, locality, collectors and data of the holotype, deposited in the tick collection of the Facultad de Ciencias Veterinarias, Universidad de Concepción, Chillán, Chile (CLZCh-UdeC 67-1 – 67-10). Ten larvae mounted in slides, two females and one male in 70% ethanol, same host, locality, collectors and data of the holotype, deposited in DPVURU, Uruguay (DPVURU - 878). Three larvae mounted in slides, one female and three males in 70% ethanol, same host, locality, collectors and data of the holotype, deposited in the “Coleção Nacional de Carrapatos” (CNC), University of São Paulo, São Paulo, Brazil (CNC-3256, -3262). Two larvae mounted in slides, host: *Callopistes maculatus* Gravenhorst 1838 (Squamata: Teiidae). Las Chinchillas National Reserve (31°28'S, 71°03'W), Coquimbo Region, Chile, coll. S. Muñoz-Leal and D. González-Acuña, December 12, 2010, deposited in DPVURU, Uruguay (DPVURU - 878).

Etymology: the species is named for the type locality, which is situated in the core of Atacama Desert.

2.3.2. Analysis of mitochondrial 16S rDNA sequences and PCA

DNA from the female and larvae from PANP yielded identical 16S rDNA sequences (GenBank KT894587). On the other hand, the sequence of the larva collected in LCNP (GenBank KT894586) was 95.7% (408/426-bp) identical to the PANP sequence. The Bayesian tree inferred from mitochondrial 16S rDNA sequences of some Neotropical Argasidae are presented in Fig. 6. The two haplotypes of *O. atacamensis* formed a single clade (bootstrap 100%) grouping within a larger clade (99% bootstrap) composed by *O. puertoricensis*, *O. guaporensis*, *O. rioplatensis*, and *O. hasei*. Pairwise differences of these species with *O. atacamensis* from PANP and LCNP sequences were respectively as follow: 10.3/12.3% with *O. rioplatensis*, 13.8/14.3% with *O. hasei*, 12.4/14.2% with *O. puertoricensis*, and 15.3/17.3% with *O. guaporensis*. For the rest of the *Ornithodoros* spp. included in the phylogenetic analysis the difference was higher than 12.4%.

The results of the PCA for the morphological characters showed a clear difference between *O. atacamensis* and the remaining species included in the analysis (Fig. 7). The first principal component, which in this case explains the 52.24% of the total variance, is almost fully loaded with the following characters: central setae, circumanal setae and hypostome length. On the other hand the second component explains a 27.82% of the total variance, and heavier characters are represented by dorsal plate width and length, postcoxal setae and hypostome width.

Figure 6. Bayesian tree inferred from mitochondrial 16S rDNA partial sequences. Numbers represent the Bayesian Posterior Probability (%). Abbreviations: *An.*, *Antricola*; *Ar.*, *Argas*; *N.*, *Nothoaspis*; *O.*, *Ornithodoros*; *Ot.*, *Otobius*. GenBank accession numbers of each sequence are indicated in parenthesis. *Ornithodoros atacamensis* is indicated in bold.

Figure 7. Principal components analysis using 41 morphological characters of *O. atacamensis* (ATA 1-10), *O. guaporensis* (GUA 1-10), *O. talaje* (TAL 1-5), *O. rioplatensis* (RIO 1-10), *O. puertoricensis* (PUE 1-10) and *O. hasei* (HAS 1-5) using the features detailed in Table 1. Each point constitutes the position of each measured specimen on the reduced space.

2.4. DISCUSSION

2.4.1. Species relationships

Larvae of *O. atacamensis* are phenotypically closely related to Neotropical ticks of the *Ornithodoros talaje* species group: *O. puertoricensis*, *O. rioplatensis*, *O. talaje* s. s., *O. guaporensis* and *O. hasei* (Venzal et al. 2008, Nava et al. 2013). However, larvae of *O. atacamensis* and *O. talaje* s. s. can be separated from *O. guaporensis*, *O. rioplatensis*, *O. puertoricensis* and *O. hasei* by the total number of dorsal setae: 17 in *O. atacamensis* and *O. talaje*, 18 in *O. puertoricensis*, 19 in *O. hasei*, and 20 in *O. guaporensis* and *O. rioplatensis*. Larvae of *O. atacamensis* can be differentiated from *O. talaje* s. s. by the three following characters: body size of the unengorged larvae of *O. atacamensis* (0.761-0.834 mm long and 0.438-0.499 mm wide) larger and wider than *O. talaje* s. s. (0.438-0.499 mm long and 0.325-0.395 mm wide); dorsal plate longer in *O. atacamensis* (0.238-0.318 mm), and for the presence of five pairs of central setae on the dorsal idiosoma in *O. atacamensis* rather than four in *O. talaje* s. s. These morphological and morphometrical differences were supported by the PCA analysis.

Ornithodoros atacamensis should also be compared with *Ornithodoros microlophi* Venzal, Nava and González-Acuña 2013 and *Ornithodoros lahillei* Venzal, González-Acuña and Nava 2015, two soft ticks associated with reptiles (at least larval stages) occurring in the same localities but not in the same hosts from which *O. atacamensis* larvae were collected. Larvae of *O. microlophi* can be easily differentiated from *O. atacamensis* by the presence of six pairs of sternal setae, and from larvae of *O. lahillei* by the absence of the postcoxal setal pair.

Adults of *O. atacamensis* are phenotypically similar to mature stages of other representatives of the *O. talaje* species group, and according to Venzal et al. (2008), their morphological characters are not suitable to separate the species of this complex. However, by the comparison of unengorged female specimens, *O. atacamensis* can be clearly differentiated, since it lacks thick lateral margins anteriorly converging in a sharpened angular apex, a morphological feature that can be

notably recognized in other representatives of the group such as *O. guaporensis*, *O. puertoricensis* and *O. rioplatensis* (Endris et al. 1989, Venzal et al. 2008, Nava et al. 2013) (Fig. 8).

Figure 8. Dorsal and lateral views of adult females of *Ornithodoros atacamensis* (A, B), *O. guaporensis* (C, D), *O. puertoricensis* (E, F) and *O. rioplatensis* (G, H). Scale bar of all the images is equivalent to 2 mm.

Instead of these characteristics, the idiosoma of *O. atacamensis* presents thin lateral margins slightly raised and converging in a slightly pointed apex. Adults of *O.*

talaje s. s. are similar to *O. atacamensis*, as both ticks share an oval body, lacking of an abrupt pointed apex in the anterior portion. However, *O. talaje* s. s. has an idiosoma with a flattened posterior margin (Cooley and Kohls 1944) which, in contrast, is rounded in *O. atacamensis*. On the other hand, *O. hasei* has also been recognized as another Neotropical representative of the *O. talaje* species group (Nava et al. 2013) and in this way it also should be compared with *O. atacamensis*. The description of mature stages of *O. hasei* was presented by Matheson (1935), including drawings of the hypostome, palpi, tarsi, chelicerae and a sole picture of an adult of unspecified sex. Although the original description is explicative, it is difficult to associate the described morphological characters, particularly those of the dorsal and ventral idiosoma with the available photograph of the *O. hasei* adult. Consequently, making a reliable comparison of these characters with *O. atacamensis* is hardly possible. However, a difference between the two species can be easily observed based on morphometric data: adults of *O. hasei* are smaller (3.4 mm long – 2 mm wide) than *O. atacamensis* mature stages (female: 5.3 – 6.7 mm long, 3.2 – 3.9 mm wide; male: 4.6 – 5.4 mm long, 2.8 – 3.3 wide).

In order to gain a better understanding of the morphological relationships within the *O. talaje* species group, future studies should consider the redescription of *O. hasei* and *O. talaje* s. s. adults.

2.4.2. Phylogenetic analyses

Morphological similarities of *O. atacamensis* with the *O. talaje* species group were also corroborated by phylogenetic analyses, in which the mitochondrial 16S rDNA sequence of *O. atacamensis* grouped basal to a clade composed by corresponding sequences of the *O. talaje* species group, with the exception of *O. talaje* s. s., for which no sequence of 16S rDNA is available.

Larvae of *O. atacamensis* from PANP and LCNP are morphologically identical; however, their mitochondrial 16S rRNA sequences were 4.3% divergent. Both tick populations (PANP and LCNP) are separated by a large area of the coastal Atacama Desert (≈ 215 km), and considering that their hosts (small reptiles) have low vagility

(Fox and Shipman 2003), the encounter between them is likely impossible to occur. In this sense, the lack of gene flow between isolated populations of *O. atacamensis* might be leading to the creation of unique haplotypes of mitochondrial 16S rRNA gene in each population. Although with the evidence of the present study this is an *a priori* conclusion, analyses performed in other congeneric ticks, inhabiting similar dry environments, have shown a high genetic variability in mitochondrial genes. This is the case of populations of *Ornithodoros sonrai* species group in desert areas of Western Africa, in which mitochondrial 16S rRNA gene haplotypes can reach up to 15% of genetic divergence (Vial et al. 2006). Considering the ecologic scenario where it occurs, the morphological similarities and observed genetic divergence between its populations, *O. atacamensis* it is likely to be a complex of sibling species. However, studies including the analysis of more genes and specimens from other localities, such as LCNR, are needed to prove this hypothesis. Until more evidence does not exist, the two analysed tick populations, PANP and LCNP, should be considered as one species.

2.4.3. Biological observations

The larva of *O. atacamensis* was collected in three different arid environments of northern Chile (PANP, LCNP and LCNR), always in association with reptiles. Other vertebrates, such as rodents and birds, were also captured and examined for ticks in these same three localities and none of them was parasitized by *O. atacamensis*. Therefore, we suggest that at least larval stages of this new species of soft tick might be mainly associated with reptiles. Adults of *O. atacamensis* were also found in reptile-frequented rock crevices in PNPA, which reinforce our proposition about host specificity. In addition, in the laboratory we were unable to feed immature stages on rabbits, mice, guinea pigs and chickens. Finally, if we consider the geographic distribution of the reptiles parasitized with *O. atacamensis*, then this soft tick could be occurring within a larger latitudinal range, towards the north and also to the south of the localities included in this study.

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3. DESCRIPTION OF TWO NEW BURROW-DWELLER SPECIES OF SOFT TICKS (ACARI: ARGASIDAE: *Ornithodoros*) FROM CENTRAL CHILE

3.1. INTRODUCTION

Soft ticks (Argasidae) of genus *Ornithodoros* Koch are currently represented by 129 species, with 61 of them occurring in the Neotropical Zoogeographic Region (Guglielmone et al. 2003, Labruna et al. 2016, Muñoz-Leal et al. 2017a). An important number of *Ornithodoros* ticks are burrow-dweller parasites of small mammals (Durden 2006), and are well adapted to survive in arid environments (Hoogstraal 1985, Oliver 1989). In South America, *Ornithodoros* ticks recorded in association with rodents inhabiting arid ecosystems include three recently described taxa: *Ornithodoros rietcorreae* Labruna, Nava & Venzal 2016 (Labruna et al. 2016), *Ornithodoros quilinensis* Venzal, Nava & Mangold 2012 (Venzal et al. 2012), and *Ornithodoros xerophylus* Venzal, Nava & Mangold 2015 (Venzal et al. 2015).

In Chile, the genus *Ornithodoros* is currently composed by seven species (Muñoz-Leal et al. 2016, Nava et al. 2017), all described for northern and central regions of the country, and always associated either to reptiles (Venzal et al. 2008, 2013, 2015, Muñoz-Leal et al. 2016) or seabirds (González-Acuña and Guglielmone 2005, González-acuña et al. 2008). Ecosystems in central Chile (roughly 29° - 37° S) are shaped by an arid Mediterranean regime (Di Castri and Hajek 1976), with several endemic and non-endemic rodent species composing a great part of its extant

vertebrate diversity (Iriarte 2008). This conjunct of biotic and abiotic features constitutes ideal conditions in which a hidden diversity of *Ornithodoros* ticks might occur, and prompted the prospections of the current study. By means of a morphological and molecular approach, two new species of *Ornithodoros* are herein described upon material collected inside rodent burrows in two localities of central Chile.

3.2. MATERIAL AND METHODS

3.2.2. Tick collection

Ticks of *Ornithodoros* genus were collected sieving substrate from inside burrows in semi-desert areas located within Bosque de Fray Jorge National Park (BFJNP, 30°33'S; 71°37'W; elevation 519 m), and in Las Chinchillas National Reserve (LCNR, 31°30'S; 71°06'W; elevation 551 m). In addition, four *Octodon degus* Molina 1978 rodents (Rodentia: Octodontidae) were captured using Sherman-like traps and examined for tick parasitism in LCNR (Figure 1). While twelve larvae collected from *O. degus* were preserved in 70-90% ethanol, four males and eight females collected from burrows of unknown host in BFJNP, and six males and eight females collected inside *O. degus*-burrows in LCNR were brought alive to the laboratory and placed inside an incubator at 25 °C and 80% of relative humidity. One female from LCNR and two females from BFJNP laid eggs. Hatched larvae were killed in hot water ($\approx 60^{\circ}\text{C}$) and preserved in 70% ethanol for morphological and molecular analyses. The capture of rodents was approved by the "Servicio Agrícola and Ganadero" (SAG, permit 6007/2014), and the "Corporación Nacional Forestal" (CONAF) authorized the prospections within BFJNP and LCNR (permit 033/2014).

Figure 1. Localities where *Ornithodoros* ticks were collected: (A) arid ecosystem in Central Chile (30°S); (B) sieved sand taken from inside an *Octodon degus*-burrow (LCNR); (C) tick recovered from sieved substrate (BFJNP), and (D) lateral view of an *O. degus* adult (LCNR).

3.2.3. Morphological analyses

A total of 22 unfed larvae obtained from females collected in BFJNP, 13 unfed larvae obtained from LNCR-collected females, and 12 of 31 engorged larvae collected from *O. degus* were clarified in a 20% KOH solution and slide-mounted using Hoyer's medium. Slides were photographed with an Olympus DP70 camera implemented in an Olympus BX40 optical microscope, and measured with the software Image-Plus Pro v5.1. Females and males were flushed with distilled water, dried with absorbent paper, photographed in a SteREO Discovery V12 stereomicroscope, and measured using the software ZEN 2 pro. All measurements

are given in millimetres (mm), with the mean followed by the standard deviation and range in parentheses. Terminology for larval chaetotaxy and measurements followed (Venzal et al. 2008). Cooley and Kohls (1944) definitions were used for the description of adult characters. Subgeneric classification followed Clifford et al. (1964). External morphology of adult specimens was compared with available descriptions of Neotropical species (Aragão 1931, Barros-Battesti 2012), and also with ticks deposited at the “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (CNC) of the Faculty of Veterinary Medicine of the University of São Paulo, Brazil, using the following specimens: *Ornithodoros rostratus* Aragão 1911 from Brazil (CNC-941), *Ornithodoros brasiliensis* Aragão 1923 from Brazil (CNC-1533), and *Ornithodoros furcosus* Neumann 1908 from Peru (CNC-3585).

3.2.4. Principal component analysis

A principal component analysis (PCA) based on Pearson correlation matrix was performed for 44 morphometric variables of unengorged larvae in order to discriminate the relationship between phenotypically and ecologically related species. To this effect, measurements of *O. quillinensis*, *O. xerophylus* and *Ornithodoros lahillei* Venzal, González-Acuña & Nava 2015 published in Venzal et al. (2015) were included.

3.2.5. Molecular tools

One female and one male from each locality, two unfed larvae (one hatched from a female of BFJNP and one from LCNR), and two engorged larvae collected from *O. degus*, were individually submitted to DNA extraction using the Guanidine Isothiocyanate technique (Sangioni et al. 2005). A conventional PCR was performed using the primers 3'- CCGGTCTCAACTCAGATCAAGT-5' (forward) and 3'- GCTCAATGATTTTTTAAATTGCTGT-5' (reverse), which amplify a ≈460-bp fragment of the tick mitochondrial 16S rRNA gene, as previously described (Mangold et al.

1998). Expected size products were purified and sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, CA) with the same primers used for the PCR. Obtained sense and antisense sequences were assembled and primer-trimmed with Geneious R9 (Kearse et al. 2012), and subsequently submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) in order to infer closest similarities with other *Ornithodoros* spp. available in GenBank.

3.2.6. Phylogenetic analysis

Obtained sequences were aligned with sequences of other species available in GenBank using Clustal X (Thompson et al. 1997), and were manually adjusted using GeneDoc (Nicholas et al. 1997). Two phylogenetic analyses were performed with the resulted alignment. A Maximum Parsimony (MP) tree was constructed using PAUP version 4.0b10 (Swofford 2002) with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. Subsequently, a Bayesian analysis (BA) was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences from *Ixodes holocyclus* Neumann 1899 and *Ixodes uriae* White 1852 were used as out-group. Accessions numbers of all sequences are shown in the phylogenetic tree.

3.3. RESULTS

3.3.1. Descriptions

Ixodida Leach, 1815

Argasidae Canestrini, 1890

Ornithodoros Koch, 1844

3.3.1.1. *Ornithodoros* n. sp. 1

Larva (Figure 2, and 8A, B, C, and F; measurements based in 10 unfed slide-mounted specimens)

Body. Small. Idiosoma subcircular. Length including capitulum: 0.552 ± 0.010 (0.532-0.568), and not including capitulum: 0.326 ± 0.011 (0.309-0.347), width: 0.333 ± 0.013 (0.312-0.351).

Dorsum. Dorsal plate oval, well defined, wider in the base, length 0.232 ± 0.005 (0.226-0.243), width: 0.198 ± 0.009 (0.182-0.208). Dorsal surface provided with 14 pairs of setae, 7 anterolateral, 3 central and 4 posterolateral. Anterolateral setae (Al): Al₁ length 0.036 ± 0.003 (0.033-0.043), Al₂ length 0.040 ± 0.003 (0.037-0.045), Al₃ length 0.045 ± 0.003 (0.041-0.050), Al₄ length 0.048 ± 0.002 (0.042-0.050), Al₅ length 0.056 ± 0.002 (0.052-0.058), Al₆ length 0.054 ± 0.002 (0.051-0.057), Al₇ length 0.070 ± 0.004 (0.064-0.078). Central setae (C): C₁ length 0.039 ± 0.003 (0.036-0.044), C₂ length 0.067 ± 0.003 (0.063-0.073), C₃ length 0.071 ± 0.004 (0.065-0.079). Posterolateral setae (Pl): Pl₁ length 0.075 ± 0.003 (0.071-0.080), Pl₂ length 0.074 ± 0.002 (0.071-0.079), Pl₃ length 0.068 ± 0.003 (0.063-0.074), Pl₄ length 0.067 ± 0.003 (0.062-0.073).

Venter. Ventral surface provided with 7 pairs of setae plus 1 pair on anal valves, posteromedian seta present and located near anal plate. Anal valves long, posteriorly protruding the anal area and leaf-shaped. Three pairs of sternal setae (St): St₁ length 0.033 ± 0.002 (0.029-0.036), St₂ length 0.032 ± 0.002 (0.029-0.034), St₃ length 0.032 ± 0.003 (0.027-0.036); three pairs of circumanal setae (Ca): Ca₁ length 0.029 ± 0.004 (0.023-0.035), Ca₂ length 0.033 ± 0.002 (0.030-0.037), Ca₃ length 0.042 ± 0.003 (0.038-0.049); posteromedian setae (PM) length 0.034 ± 0.004 (0.027-0.042), postcoxal setae (Pc) length 0.018 ± 0.003 (0.014-0.023).

Capitulum. Basis capituli pentagonal, posterior margin softly curved, with a slight concavity in the median portion of some slide-mounted specimens. Length from posterior margin of basis capituli to posthypostomal setae Ph₁ 0.074 ± 0.004 (0.065-0.078), length from posterior margin of basis capituli to insertion of hypostome 0.091 ± 0.007 (0.079-0.104), length from posterior margin of basis capituli to apex of hypostome 0.269 ± 0.006 (0.257-0.278), width 0.149 ± 0.007 (0.138-0.158). Two pairs of posthypostomal setae; Ph₁ length 0.012 ± 0.002 (0.009-0.014), Ph₂ length 0.041 ± 0.006 (0.028-0.052), distance between Ph₁ setae 0.012 ± 0.002 (0.009-0.014), distance between Ph₂ setae 0.062 ± 0.003 (0.058-0.067). Palpi: total length 0.199 ± 0.004 (0.193-0.206), segmental length/width from I-IV: (I) 0.037 ± 0.002 (0.033-0.041) / 0.024 ± 0.002 (0.022-0.027), (II) 0.070 ± 0.003 (0.064-0.073) / 0.026 ± 0.001 (0.023-0.028), (III) 0.070 ± 0.002 (0.067-0.073) / 0.024 ± 0.001 (0.022-0.025), (IV) 0.036 ± 0.002 (0.034-0.042) / 0.014 ± 0.001 (0.013-0.015). Setae number on palpal articles I-IV: (I) 0, (II) 4, (III) 5, (IV) 9.

Hypostome. Length from Ph₁ to apex 0.196 ± 0.003 (0.190-0.202), length from insertion of hypostome in basis capituli to apex 0.171 ± 0.006 (0.160-0.182), width in medial basis portion of hypostome 0.038 ± 0.001 (0.037-0.040), width in basis portion of hypostome 0.038 ± 0.001 (0.037-0.039). Denticles arising directly from the basis. Blunt apically. Dentition formula 2/2 in all its extension, file 1 with 21 to 22 denticles (typically 21) and file 2 with 20 to 21 (typically 20).

Legs. Tarsus I length 0.136 ± 0.003 (0.130-0.140), tarsus I width 0.046 ± 0.001 (0.044-0.048). Setal formula of tarsus I: 1 pair apical (A), 1 distomedian (DM), 5 paracapsular (PC), 1 posteromedian (PM), 1 pair basal (B), 1 pair apicoventral (AV), 1 pair midventral (MV), 1 pair basiventral (BV), and 1 pair posterolateral (PL).

Capsule of Haller's organ: irregular in shape, without reticulations. Coxa I, II, and III provided with one small ventral spur projecting towards the trochanter and with two ventral setae. Bifid claws and pulvillus present.

Figure 2. *Ornithodoros* n. sp. 1 Drawing of holotype larva: (A) dorsal, and (B) ventral views; (C) tarsus I.

Female (Figure 3A, B, C, D, F, and G; measurements based on eight females)

Body. Oval, 4.296 ± 0.533 (3.586 – 5.122) long, 2.723 ± 0.279 (2.3 – 3.151) wide. Posterior margin rounded, lateral margins subparallel, anteriorly converging to

a small angular apex. Dorsum covered by mammillae of variable size and shape, larger with a truncated conical shape in the posterior margin and smaller with an irregular shape in the anterior margin and rest of the dorsum. Some mammillae provided of a short seta. Middle of the idiosoma with bilaterally symmetrical depressed regions provided in the middle with barely notable group of disks, below the level of mammillae, not present at the margins. Supracoxal fold small and thin. Lateral suture absent. Venter: little irregular mammillae covering the ventral surface with few small discs only present in preanal and median-postanal grooves. Dorso-ventral groove absent. Preanal groove straight above anus. Coxal fold pronounced. Genital area surrounded by a depressed micromamillated area and located between posterior ends of coxae I; anterior labia slightly larger than posterior. Anus surrounded by a ring of irregular mammillae in its margin; Intersection of median-postanal and postanal groove with a pair of glabrous folds as pictured. Spiracular plate small and above coxa IV.

Capitulum. Situated ventrally to a small hood. Cheeks small and elongate, not covering the palps, hypostome or mouthparts. Basis capituli rectangular micro-mamillated, 0.199 ± 0.027 ($0.166 - 0.241$) long, 0.361 ± 0.027 ($0.295 - 0.372$) wide, with one pair of long posthypostomal setae, one pair (some specimens with two) of postpalpal setae approximately half of the size of the posthypostomal setae. Hypostome with dental formula 2/2; small crenulations apically.

Legs. Short, entirely micro-mamillated with exception for coxae I–IV, which are partially mamillated (coxa IV almost unmamillated). Coxae I and II separated by a mamillated integument portion; coxae II to IV contiguous. Size of coxae in decreasing order: IV, III, II, I. Tarsi narrow without humps and protuberances.

Male (Figure 3E, measurements based in four specimens)

Similar to the female except in the genital area. Body 3.006 ± 0.248 ($2.799 - 3.307$) long, 1.889 ± 0.156 ($1.688 - 2.047$) wide. Basis capituli 0.178 ± 0.011 ($0.168 - 0.194$) long, 0.272 ± 0.016 ($0.251 - 0.284$) wide. Genital flap smooth and without crenulations.

Figure 3. *Ornithodoros* n. sp. 1 holotype female (fem.) and allotype male. (A) Dorsal view (fem.), (B) ventral view (fem.), (C) capitulum (fem), (D) fem. genital area, (E) male genital area, (F) anterior part of the dorsum, and (G) posterior part of the dorsum.

Taxonomical summary

Type host: *Octodon degus* Molina 1782 (Rodentia: Octodontidae).

Type locality: Las Chinchillas National Reserve (31°30'S; 71°06'W), Coquimbo Region, Chile.

Type specimens: the type series used for the description of this new species has been separated for future deposition in the allotments of the “Coleção Nacional de Carrapatos Danilo Saraiva” (CNC), University of São Paulo, São Paulo Brazil; USNTC, Georgia Southern University, Statesboro, USA; DPVURU, Universidad de la República, Salto, Uruguay; IBSP, Instituto Buntantan, São Paulo, Brazil; and CLZCh UdeC, Chillán, Chile.

3.3.1.2. *Ornithodoros* n. sp. 2

Larva (Figure 4, and 8D, E; measurements based on 20 unfed slide-mounted specimens)

Body. Idiosoma subcircular. Length including capitulum: 0.846 ± 0.015 (0.820 - 0.871), and not including capitulum: 0.620 ± 0.011 (0.610 - 0.642), width: 0.579 ± 0.013 (0.559 - 0.606).

Dorsum. Dorsal plate sub-rectangular, irregular in shape, length 0.170 ± 0.007 (0.156 - 0.177), width: 0.134 ± 0.007 (0.122 - 0.143). Dorsal surface provided with 13 pairs of setae, seven anterolateral, three central and three posterolateral. Anterolateral setae (Al): Al₁ length 0.131 ± 0.006 (0.121 - 0.139), Al₂ length 0.141 ± 0.004 (0.135 - 0.146), Al₃ length 0.126 ± 0.010 (0.107 - 0.146), Al₄ length 0.119 ± 0.006 (0.109 - 0.128), Al₅ length 0.116 ± 0.005 (0.111 - 0.128), Al₆ length 0.107 ± 0.006 (0.098 - 0.114), Al₇ length 0.118 ± 0.005 (0.110 - 0.129). Central setae (C): C₁ length 0.090 ± 0.005 (0.082 - 0.098), C₂ length 0.105 ± 0.009 (0.090 - 0.118), C₃ length 0.115 ± 0.005 (0.109 - 0.122). Posterolateral setae (Pl): Pl₁ length 0.142 ± 0.008 (0.131 - 0.158), Pl₂ length 0.148 ± 0.007 (0.138 - 0.158), Pl₃ length 0.143 ± 0.013 (0.119 - 0.166). Accessory Pl, Al and C setae present in some specimens. One pair of small lateral grooves, posteriorly projected, located between setae Al₇ and Pl₁. Presence of a pronounced short depression, not forming a groove, in medial portion of posterior margin. Several pores spread along the idiosoma, some of them bilaterally symmetrical (included in the drawings).

Venter. Ventral surface provided with seven pairs of setae plus one pair on anal valves, posteromedian seta present. Three pairs of sternal setae (St): St₁ length 0.042 ± 0.004 (0.036 - 0.049), St₂ length 0.040 ± 0.002 (0.037 - 0.045), St₃ length 0.040 ± 0.006 (0.033 - 0.050); three pairs of circumanal setae (Ca): Ca₁ length 0.044 ± 0.003 (0.040 - 0.048), Ca₂ length 0.057 ± 0.002 (0.053 - 0.061), Ca₃ length 0.081 ± 0.003 (0.075 - 0.086); posteromedian setae (Pm) length 0.074 ± 0.004 (0.066 - 0.081), postcoxal setae (Pc) length 0.062 ± 0.005 (0.053 - 0.061).

Capitulum. Ventral: basis capituli sub-rectangular. Length from posterior margin of basis capituli to posthypostomal setae Ph_1 0.141 ± 0.006 (0.136 - 0.156), length from posterior margin of basis capituli to insertion of hypostome 0.159 ± 0.008 (0.148 - 0.176), length from posterior margin of basis capituli to apex of hypostome 0.314 ± 0.008 (0.306 - 0.329), width 0.197 ± 0.006 (0.191 - 0.207). Two pairs of posthypostomal setae, Ph_1 length 0.051 ± 0.003 (0.044 - 0.054), Ph_2 length 0.033 ± 0.002 (0.030 - 0.038), distance between Ph_1 setae 0.028 ± 0.002 (0.025 - 0.031), distance between Ph_2 setae 0.096 ± 0.005 (0.087 - 0.102). Chelicerae thick with a wrinkled surface and abundant crenulations near the insertion of digits. *Dorsal:* conical in shape, with a straight base. *Palpi:* total length 0.203 ± 0.003 (0.200 - 0.211), segmental length/width from I-IV: (I) 0.050 ± 0.004 (0.042 - 0.054) / 0.052 ± 0.003 (0.047 - 0.058), (II) 0.059 ± 0.004 (0.053 - 0.066) / 0.040 ± 0.002 (0.038 - 0.043), (III) 0.058 ± 0.005 (0.051 - 0.067) / 0.038 ± 0.001 (0.036 - 0.040), (IV) 0.055 ± 0.004 (0.046 - 0.060) / 0.029 ± 0.001 (0.027 - 0.030). Setae number on palpal articles I-IV: (I) 0, (II) 4, (III) 5, (IV) 9. Article I with a knife-edge flange in its median side.

Hypostome. Length from Ph_1 to apex 0.174 ± 0.003 (0.169 - 0.179), length from apex to inferior toothed portion 0.130 ± 0.005 (0.122 - 0.138), insertion of hypostome in basis capituli to apex 0.151 ± 0.009 (0.135 - 0.160), width in medial basis portion of hypostome 0.059 ± 0.004 (0.055 - 0.067), width in basis portion of hypostome 0.063 ± 0.006 (0.054 - 0.070); first proximal 1/4 without denticles. Blunt apically. Dentition formula 2/2 in all its extension, file 1 with 8 - 9 denticles and file 2 with 5 - 6. Corona with 2 - 3 crenulations per side. Apex notched.

Legs. Tarsus I length 0.204 ± 0.004 (0.198 - 0.211), width 0.064 ± 0.002 (0.062 - 0.068), with a pronounced subapical dorsal protuberance. Bifid claws and pulvillus present. Setal formula of tarsus I: 1 pair apical (A), 1 short distomedian (DM), 5 paracapsular (PC), 1 posteromedian (PM), 1 basal (B), 1 pair apicoventral (AV), 1 pair midventral (MV), 1 basiventral (BV), and 1 pair posterolateral (PL). Capsule of Haller's organ: sub-oval in shape, without reticulations. Six little thick setae between Haller's organ and DM seta. Coxa I, II, and III with one small ventral spur projecting towards the trochanter. Each coxa provided with two ventral setae.

Figure 4. *Ornithodoros* n. sp. 2. Drawing of holotype larva: (A) dorsal, and (B) ventral views; (C) tarsus I.

Female (Figure 5A, B, and C; measurements based on six specimens)

Body. Oval, 5.319 ± 0.715 (4.507 – 6.289) long, 3.605 ± 0.588 (2.752 – 4.407) wide. Posterior and anterior margins rounded, hood barely visible from above. Lateral margins subparallel. Dorsum: integument covered by numerous mammillae moderate and uniform in size. Each mammilla regularly spaced, sub-oval in shape, with a convex and smooth surface. Truncated conical elevations spread among mammillae and provided with a seta, shorter and fewer in the posterior portion of the idiosome,

more abundant and longer in the anterior margin and supracoxal fold. Supracoxal fold broadly thick, extending from above coxa IV towards the comerostome. Middle of the idiosoma with small discs below the level of mammillae, and not present at the margins. Lateral suture absent. Venter: round and smooth mammillae covering the ventral surface with few small discs only present in preanal and median-postanal grooves. Dorso-ventral groove pronounced and visible in dorsal view. Coxal grooves present, separated by a median depression in the integument. Genital area located between the intersections of coxae I and II. Anterior labia small, projecting posteriorly forming an integument that surrounds a larger posterior labia provided with a median depression. Anus circular surrounded by a ring of irregular mammillae in its margin; anal plates provided with several setae. The intersection of median-postanal and postanal grooves divides the paired glabrous organs in four quadrants. Spiracular plate small and above coxa III.

Capitulum. Capitulum situated ventrally to a broadly rounded hood. Cheeks absent. Basis capituli subrectangular, micro-mammillated, 0.282 ± 0.042 ($0.244 - 0.360$) long, 0.635 ± 0.045 ($0.575 - 0.684$) wide, with one pair of long posthypostomal setae, pair of postpalpal setae absent. Hypostome with dental formula 2/2; apically notched with small crenulations. *Legs:* entirely micro-mammillated with exception of coxae I to IV, which are covered with a wrinkled integument. Size of coxae from larger to smaller: I, II, III, and IV. Coxae I and II slightly separated, coxae II to IV contiguous. All tarsi provided with a conspicuous subapical dorsal protuberance. Tarsus I with two dorsal humps, the proximal longer than the distal (Fig. 10M). Tarsus II with two prominent dorsal humps, the proximal larger than the distal. Tarsus III, provided with humps of similar disposition and morphology as tarsus II, but smaller in size; tarsus IV longer than the previous segments, without humps (Fig. 10N, O, and P)

Male (Figs 5D – measurements based on four specimens)

Similar to the female except in the genital area. Body 4.261 ± 0.245 ($3.921 - 4.506$) long, 2.732 ± 0.261 ($2.401 - 2.984$) wide. Basis capituli 0.214 ± 0.015 ($0.201 - 0.235$) long, 0.502 ± 0.067 ($0.408 - 0.557$) wide. Genital flap without crenulations.

Figure 5. Micrographs of female and male *Ornithodoros* n. sp. 2: (A) female dorsal view, (B) female ventral view, (C) female genital aperture, and (D) male genital aperture.

Taxonomic summary

Type locality: Bosque de Fray Jorge National Park (31°30'S; 71°06'W), Coquimbo Region, Chile.

Type specimens: the type series used for the description of this new species has been separated for future deposition within the allotments of the “Coleção Nacional de Carrapatos Danilo Saraiva” (CNC), University of São Paulo, São Paulo Brazil; USNTC, Georgia Southern University, Statesboro, USA; DPVURU, Universidad de la República, Salto, Uruguay; IBSP, Instituto Buntantan, São Paulo, Brazil; and CLZCh UdeC, Chillán, Chile.

3.3.2. PCA

By the PCA performed with 44 morphological variables, *Ornithodoros* n. sp. 1 clearly differs from *O. quilinensis*, *O. lahillei* and *O. xerophylus* (Figure 6). The first two components explain the 70.67 of the total variance. The first one explained the 39.09% of the total variance and is principally loaded with the following characters: length of central setae length (C_1), and circumanal setae (Ca_2 and Ca_3); distance between both Ph_1 setae, and apical dental formula. The second component explains a 31.58% of the total variance, and the heavier characters are represented by ventral setae, circum-anal setae (Ca_1), postcoxal setae and palpal length (article IV).

Figure 6. Principal component analysis: *Ornithodoros* n. sp. 1 (OCT 1–10), *O. lahillei* (LAH 1–10), *O. quilinensis* (QUI 1–10), and *O. xerophylus* (XER 1–10). Each point constitutes the position of each measured specimen on the reduced space.

3.3.3. Analysis of the mitochondrial 16S rDNA sequences and phylogenetic tree

For each species, identical mitochondrial 16S rDNA sequences were retrieved from adults and larvae submitted to DNA extraction. Pairwise comparisons using BLAST pointed *O. quilinensis* (JN255575) and *Ornithodoros peropteryx* Kohls, Clifford & Jones, 1969 (KC493651) as most similar species with 86.8% (363/418-bp) and 84.8% (364/429-bp) of identity with *Ornithodoros* n. sp. 1 and *Ornithodoros* n. sp. 2, respectively. Under high bootstrap and posterior probability values, the phylogenetic analysis of the 16S rDNA fragments supports that *Ornithodoros* n. sp. 1 clusters within a monophyletic group conformed by *O. quilinensis*, *O. xerophylus* and an unspecified *Ornithodoros* sp. from Bolivia. With a similar high dual support,

Ornithodoros n. sp. 2 grouped as a sister taxon of a clade composed by *O. brasiliensis* and *O. rostratus* (Figure 7).

Figure 7. Phylogenetic tree inferred from tick mitochondrial 16S rDNA partial sequences. Sequences of *Ornithodoros n. sp. 1* and *Ornithodoros n. sp. 2* are highlighted in bold. GenBank accession numbers are indicated between parentheses.

3.4. DISCUSSION

3.4.1. Species relationships

3.4.1.1. *Ornithodoros* n. sp. 1

Larva. From an ecological point of view, *Ornithodoros* n. sp. 1 has not yet any similar species to be compared, since it constitutes the first soft tick to be described as a parasite of a Chilean native rodent. For instance, two species that deserves comparison would be *Ornithodoros* n. sp. 2 and *O. lahillei*. Five significant differences strikingly separate *Ornithodoros* n. sp. 2 from *Ornithodoros* n. sp. 1: body size, dorsal plate shape, hypostome conformation, tarsal design of Haller's organ capsule and the form of anal plates. Larvae of *Ornithodoros* n. sp. 1 are almost half smaller (0.326 ± 0.011) than larvae of *Ornithodoros* n. sp. 2 (0.620 ± 0.011), and the shape of the dorsal plate is oval (egg-like) in the former, and rather subrectangular with an irregular outline in the later species (Figure 8A, D); the hypostome is thin (0.038 ± 0.001) and long (0.176 ± 0.005) in *Ornithodoros* n. sp. 1, and thick (0.063 ± 0.006) and short (0.151 ± 0.009) in *Ornithodoros* n. sp. 2 (Figure 8B, C); finally, *Ornithodoros* n. sp. 1 presents an irregularly delimited capsule of Haller's organ, and leaf-shaped anal plates (Figure 8C, F), in turn *Ornithodoros* n. sp. 2 lack these features and rather presents an oval and defined Haller's organ capsule, and anal plates without any salient structure. With *O. lahillei*, *Ornithodoros* n. sp. 1 differs in having a blunt hypostome, which is rather pointed in *O. lahillei*, and by having a pair of poxcoxal setae, which in turn are absent in *O. lahillei*.

Although in ecological terms *Ornithodoros* n. sp. 1 has not yet any other species to be compared in the western slopes of Southern South American Andes, two rodent-associated and morphologically similar species, known only from larval stages, have been described for the Chacoan Region of Argentina (Venzal et al. 2012, 2015). Remarkably, *Ornithodoros quilinensis* and *O. xerophilus* share the three following characters with *Ornithodoros* n. sp. 1: small body size, an oval and

similarly sized dorsal plate, an irregularly shaped capsule of Haller's organ, hypostome apically blunt with dentition 2/2 from apex to base, and 14 – 16 pairs of dorsal setae. However, *Ornithodoros* n. sp. 1 differs from the other two species in having a longer hypostome provided with 21 – 22 denticles in rows one and two (Figure 8B), instead of 14 – 16 denticles in *O. xeropylus* (Venzal et al. 2015), and ≈ 14 denticles for both rows in the case of *O. quilinensis* (Venzal et al. 2012). Moreover, only larvae of *Ornithodoros* n. sp. 1 are provided with leaf-shaped anal plates.

The larva of *Ornithodoros* n. sp. 1 should also be compared with *Ornithodoros rietcorreae* Labruna, Venzal & Nava, 2016, a species associated with rodents in Brazil. Both species have in common leaf-shaped anal plates; however they strikingly differ in the anatomy of the hypostome and form of the dorsal plate. In *Ornithodoros* n. sp. 1 the hypostome is provided with two rows from the apex until its insertion in the capitulum, rather than three in the first half, and then two towards the base but not reaching the insertion in *O. rietcorreae*. Likewise, *Ornithodoros* n. sp. 1 has an oval dorsal plate, which in turn is pyriform in *O. rietcorreae* (Labruna et al. 2016).

Figure 8. Optical micrographs of *Ornithodoros* n. sp. 1 and *Ornithodoros* n. sp. 2 larvae. *Ornithodoros* n. sp. 1: (A) dorsal plate, (B) hypostome, (C) tarsus I (capsule of Haller's organ with an arrow), and (F) anal plates with leaf-shaped protrusions (each one marked with an arrow). *Ornithodoros* n. sp. 2: (D) dorsal plate, and (E) hypostome.

Adults. Females and males of *Ornithodoros* n. sp. 1 must be compared with adult stages of the sympatric species *Ornithodoros atacamensis* Muñoz-Leal, Venzal & González-Acuña 2016, and the herein presented *Ornithodoros* n. sp. 2. With the first species, *Ornithodoros* n. sp. 1 differs in having wrinkled undefined mammillae and barely marked discs, instead of well defined mammillae (in a cell-like pattern) and marked discs present in females and males of *O. atacamensis* (Muñoz-Leal et al. 2016). On the other hand, mature stages of *Ornithodoros* n. sp. 1 can be easily separated from adult specimens of *Ornithodoros* n. sp. 2 since they possess cheeks, and *Ornithodoros* n. sp. 2 do not.

3.4.1.2. *Ornithodoros* n. sp. 2

Larva. The larvae of *Ornithodoros* n. sp. 2 are morphologically similar to *O. brasiliensis* and *O. rostratus*. All three larvae share an irregularly shaped dorsal plate, a dorsal surface provided with 10 pairs of dorsolateral setae and three central pairs; two rows of denticles not reaching the insertion of the hypostome in the capitulum, and large body size (≈ 0.850 mm length including capitulum) (Kohls et al. 1965, Barros-battesti 2012). Still, some slight differences allow a discrete separation at least between *Ornithodoros* n. sp. 2 and *O. rostratus*. In average larvae of *Ornithodoros* n. sp. 2 have evidently longer pairs of dorsal posterolateral setae than *O. rostratus* (0.144 and 0.089 mm respectively). Moreover, pairs of Ph1 and Ph2 setae are almost of the same size in *O. rostratus* (0.031 and 0.034 mm, respectively) (Kohls et al. 1965), however Ph1 is longer than Ph2 in *Ornithodoros* n. sp. 1 (0.051 and 0.033 mm, respectively). On the other hand, phenotypes of *Ornithodoros* n. sp. 2 and *O. brasiliensis* are extremely similar. The separation of larvae upon direct observation of the morphological characters and morphometry herein described is difficult. However, this phenotypic overlap in larval traits is utterly compensated by the differences observed in adult morphology.

Larval stages of *Ornithodoros* n. sp. 2 should also be compared with *O. furcosus*, yet at the time of the redaction of the current study, it was impossible to

examine larval specimens of this later species. However, and in the same way as with *O. brasiliensis*, this gap is convincingly filled with morphological comparisons of mature stages.

Adults. Females and males of *Ornithodoros* n. sp. 2 lack cheeks, and are provided with a dorsoventral groove. Taking into account the subgeneric classification adopted for the current species description, these features constitute taxonomically important traits matching the morphological characters shared by representatives of the subgenus *Pavloskyella* (Clifford et al. 1964). Mirroring the species owing to be compared for the separation of larval stages, the phenotype of *Ornithodoros* n. sp. 2 mature stages should be contrasted with the *Pavlovskyella* species described for South America, namely *O. brasiliensis*, *O. furcosus*, and *O. rostratus*. Adults of *Ornithodoros* n. sp. 2 adults can be separated from the above three species by the following combination of characters: 1) small in size (2.89 – 6.289 mm long), 2) body outline oval, 3) hood not visible from an above view (Figure 9A), 4) tarsi short, and 5) tibia I provided with three flattened humps (Figure 10). Moreover, these other three species are larger than *Ornithodoros* n. sp. 2, with a subrectangular body outline, the hood is visible from above, and tibiae I differ as pictured (Figure 9).

Figure 9. South American species of *Pavlovskyella* subgenus. (A) *Ornithodoros* n. sp. 2 (female), (B) *O. furcosus* (female), (C) *O. rostratus* (female), and (D) *O. brasiliensis* (male). Scale bar is equivalent to one millimetre.

Figure 10. Tibiae and tarsi I, II, III, and IV of *O. brasiliensis*, *O. furcosus*, *O. rostratus*, and *Ornithodoros* n. sp. 2. File one, two, three and four contain images of legs I, II, III and IV respectively. (A, B, C, and D) *O. brasiliensis*, (E, F, G, and H) *O. furcosus*, (I, J, K, and L) *O. rostratus*, and (M, N, O, and P) *Ornithodoros* n. sp. 2. The three flattened humps of tibia I present in *Ornithodoros* n. sp. 2 are indicated with arrows. Scale bar is equivalent to 0.5 millimetres.

3.4.2. Subgeneric classification

The criteria for subgeneric classification adopted in the current study divide the Argasidae family based in 1) morphological characters of immature and mature stages and, 2) in the comparison of ontological characters (Clifford et al. 1964, Kohls et al. 1965, Hoogstraal 1985). Briefly, morphological and developmental characters common to the subgenus *Alectorobius* Pocock 1907 include: the presence of cheeks in adults; lack of dorsoventral grooves and tarsal humps in post larval stages; larvae generally with a pyriform dorsal plate; dorsum provided with 11 - 18 pairs of dorsolateral setae, and three to five pairs central; hypostome with two or more rows of denticles, posthypostomal seta Ph₁ shorter than Ph₂; lack of a spinose area adjacent to Haller's organ capsule; and long feeding time of larvae followed by two

successive molts to the second nymphal stage without feeding. In turn, representatives of the *Pavlovskyella* subgenus share the following traits: nymphs and adults without cheeks; dorsoventral groove and tarsal humps present; larvae with an irregular dorsal plate (absent in some species); dorsum provided with 13 - 14 pairs of dorsolateral setae, and two central pairs; hypostome blunt provided with two files of denticles; posthypostomal seta Ph_1 longer than Ph_2 , presence of a spinose area adjacent to Haller's organ capsule; and each stage involving a rapid feeding behavior, with only one molt after each blood meal (Clifford et al. 1964, Kohls et al. 1965, Hoogstraal 1985).

In the current study the data obtained for each species lack the description of developmental traits in order to completely fit the criteria for subgeneric classification. While *Ornithodoros* n. sp. 1 would be considered as an *Alectorobius* species by the presence of cheeks in adults and morphological characters of larvae, until biological features of the first nymphal instar are not elucidated, a subgeneric classification would be rather speculative. Moreover, this conservative criterion should also be applied for *Ornithodoros* n. sp. 2. Still, in this case morphological evidence points its classification as a *Pavlovskyella* representative, which in turn is supported by a phylogenetic relatedness with *O. brasiliensis* and *O. rostratus*, two other neotropical ticks of this subgenus.

3.4.3. PCA

Although larvae of *Ornithodoros* n. sp. 1 are phenotypically similar with *O. quilinensis* and *O. xerophylus*, PCA comparisons were successful in separating species these three species and *O. lahillei*, as well. Apart from the evident differences in the hypostome length and the presence of leaf-shaped anal plates, the PCA pointed the length of central and circumanal setae as significant traits to separate these ecologically similar species.

3.4.4. Phylogenetic analysis

Ornithodoros n. sp. 1 formed a monophyletic group with *O. quilinensis*, which in turn grouped as a sister clade conformed by *O. xerophylus* and an *Ornithodoros* sp. from Bolivia. Excluding this last undetermined species for which morphological traits are unknown, *Ornithodoros* n. sp. 1, *O. quilinensis* and *O. xerophylus* are phenotypically similar species at least for larval stages, and all three are associated with South American rodents. If linking current phylogenetic and morphological evidence, this monophyletic group could correspond to a complex of species that diverged in association to South American rodents. This supposition is supported by previous phylogenetic analyses, in which *O. quilinensis*, *O. xerophylus* and the *Ornithodoros* sp. from Bolivia repeatedly clustered together (Venzal et al. 2012, 2015, Labruna et al. 2016, Muñoz-Leal et al. 2016, 2017b). To gain a better knowledge in this alleged group of soft ticks, future studies should reassess morphological traits and assess the phylogenetic position of other *Ornithodoros* of uncertain taxonomic status, such as *Ornithodoros aragaoi* Fonseca 1960 and *Ornithodoros davisii* Fonseca 1960 (Guglielmone et al. 2009), two species described upon de examination of nymphal and adult stages associated to rodents in Peru (Fonseca 1960).

As it would be expected by the morphological analyses of larvae and adults, *Ornithodoros* n. sp. 2 formed a monophyletic group with *O. brasiliensis* and *O. rostratus*. By the phylogenetic analysis, all three ticks share genetic identities with *Pavlosvskyella* subgenus representatives, which are supported by morphological traits of adults and larval stages as well. Still, by the time of realization of the current study, no genetic comparisons were made with *O. furcosus*, another Neotropical *Pavlovskyella*. Although this important comparison was not performed, it is noteworthy that *O. furcosus* is morphologically related to *O. brasiliensis* and *O. rostratus*, and it has never been reported in Chile. Moreover, taking into account that southernmost records for *O. furcosus* were in the Rimac river basin in Lima (Peru, $\approx 12^{\circ}\text{S}$) (Delgado 1967), located more than 2000 km towards the north of the *Ornithodoros* n. sp. 2 type locality (Chile, $\approx 30^{\circ}\text{S}$), which in turn is geographically isolated by the Atacama Desert in the north, Andes Mountains by the east and the

Pacific Ocean by the west, the probability that these two species indeed correspond to a unique taxon is rather unlikely to occur.

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4. BACTERIA OF THE GENUS *Rickettsia* ASSOCIATED WITH SOFT TICKS (ACARI: ARGASIDAE) FROM NORTHERN AND CENTRAL CHILE

4.1. INTRODUCTION

Microorganisms of genus *Rickettsia* are obligate intracellular Alpha-proteobacteria that naturally infect cells of a wide diversity of invertebrates, including several taxa of insects and ticks (Weinert et al. 2009). Supported by multilocus and complete genome phylogenetic analyses, the diversity of genus *Rickettsia* has been divided in 13 groups, some of them composed by pathogenic species with known vector-borne roles, yet a great majority represented by organisms of unknown pathogenicity and endosymbionts (Weinert et al., 2009; Kurtti et al., 2015; Murray et al., 2016). Since the discovery of rickettsial agents, the study of this lineage of bacteria has been historically biased towards hard ticks (Ixodidae) (Eremeeva and Dasch 2015), mainly because of the vector and reservoir roles that these parasites have, as they harbor pathogenic *Rickettsia* of public health relevance (Socolovschi et al., 2009; Parola et al., 2013).

In turn, rickettsiae associated with soft ticks (Argasidae) have been studied in a much lesser extent. While in the past ticks of this family were eventually used to maintain *Rickettsia* infections for transporting purposes between intercontinental research laboratories (Parker 1942), further sporadic research put in evidence that soft ticks were also naturally infected with a barely known *Rickettsia* diversity. First isolations of these bacteria from soft ticks included *Rickettsia rickettsii* in *Ornithodoros nicollei* (Mooser 1932) and *Otobius lagophilus* Cooley & Kohls 1940 (Silva-Goytia and Elizondo 1952), a *Rickettsia slovaca*-like agent from *Argas persicus* (Oken 1810)(Reháček et al. 1977) and *Rickettsia bellii* from *Argas cooleyi* Kohls & Hoogstraal 1960 and *Ornithodoros concanensis* Cooley & Kohls 1941 (Philip et al.

1983). More than twenty-five years later, isolation of novel organisms such as *Rickettsia hoogstraalii* (Mattila et al. 2007), *Rickettsia lusitaniae* (Milhano et al., 2014), “*Candidatus Rickettsia wissemannii*” (Tahir et al. 2016), and “*Candidatus Rickettsia nicoyana*” (Moreira-Soto et al. 2017), added novel representatives to soft tick-associated rickettsiae. Still, the characterization of several conserved and less-conserved rickettsial genes from soft tick-extracted DNA, has demonstrated the occurrence of new haplotypes, indicating that ticks of this family harbor an underestimated diversity of this group of bacteria (Loftis et al., 2005; Kawabata et al., 2006; Reeves et al., 2006; Pader et al., 2012; Dietrich et al., 2014a; Lafri et al., 2015; Sánchez-Montes et al., 2016; Tahir et al., 2016; Duron et al., 2017). Remarkably, some of these newly recognized agents are phylogenetically related with pathogenic species, such as the rickettsiae of the Spotted Fever Group (Cutler et al., 2006; Socolovschi et al., 2012; Tahir et al., 2016; Moreira-Soto et al., 2017).

All tick species are exclusive parasites of volant and non-volant vertebrates (Sonenshine and Roe 2014). In contrast to slow-feeding hard ticks (Ixodidae), soft ticks (Argasidae) are fast-feeding parasites, and are distributed in all regions of the globe with the exception of northern and southernmost latitudes (roughly, up to 40° in Northern and Southern Hemispheres). Within an still unsolved systematic scenario, currently adopted taxonomic scheme divides the Argasidae family into five genera, namely *Antricola* Cooley & Kohls, *Argas* Latreille, *Nothoaspis* Keirans & Clifford, *Ornithodoros* Koch, and *Otobius* Banks (Hoogstraal 1985), all of them (native or exotic) represented in the Neotropical Zoogeographic Region (Guglielmone et al. 2003). Particularly, the Chilean fauna of soft ticks is currently represented by three genera: *Ornithodoros* with seven native species, two native and one exotic *Argas*, and the exotic genus *Otobius* with one species (González-Acuña and Guglielmone 2005, Venzal et al. 2008, 2012, 2013, 2015, Krawczak et al. 2016, Muñoz-Leal et al. 2018). The diversity of Chilean soft ticks is distributed mainly in the northern half of the country (28 – 32°S), which is predominantly composed by arid environments ranging from sea level until above 4500 m highlands, and includes the core of the Atacama Desert, catalogued as one of the driest ecosystems on earth (Clarke 2006). In turn, some species extend their distribution towards Central Chile (32 – 37° S), where the climate gradually shifts to a Mediterranean regime (Di Castri and Hajek 1976), and a Coastal Range divides the valley between seashores and Andes Mountains.

Although soft ticks do harbor bacteria of the genus *Rickettsia*, it is unclear why the majority of pathogenic species is currently found mostly in hard ticks, or why some of these bacteria are naturally associated to both tick lineages. Since phylogenetic analyses support that soft ticks constitute an ancestral group in relation to hard ticks (Klompen et al., 1996; Mans et al., 2012, 2015), exploring the diversity of *Rickettsia* in ticks of the Argasidae family can be a good starting point to gain a better understanding on the evolutionary history of these bacteria among the Ixodoidea. By means of cell culture methods and the use of specific primers, in this study we attempted to isolate and molecularly characterize *Rickettsia* spp. infecting soft ticks from Chile, and inserted this data into a phylogenetic context.

4.2. MATERIALS AND METHODS

4.2.1. Study sites and tick collection

Mature and immature soft ticks were collected during August 2012, November to December 2014, December 2015, and November 2016 in ten localities of Northern and Central Chile as follows: Caleta Vitor (18°45'S; 70°20'W, elevation 23 m), Pampa del Tamarugal National Reserve (20°26'S; 69°41'W, elevation 1000 m), Santa María Island (23°26'S; 70°36'W, elevation 2 m), Ollagüe (21°13'S; 68°15'W, elevation 3709 m), Calama (22°27'S; 68°54'W, elevation 2775 m), Taltal (25°23'S; 70°30'W, elevation 9 m), Pan de Azúcar National Park (Island, 26°09'S; 70°40'W, elevation 12 m; mainland, 26°17'S; 70°38'W, elevation 35 m), Nevados de Chillán (36°52'S; 71°27'W, elevation 1549 m), and The Dead Islet at Mocha Island (38°25'S; 73°55'W, elevation 6 m) (Figure 1).

In each prospected locality the search for ticks was actively conducted during the day and with the following methodologies: 1) sifting the substrate extracted from small mammal and passerine burrows 2) examining rock crevices inside bat-inhabited caves, and 3) lifting rocks near bird and reptile resting places. While part of the ticks collected in Pan de Azúcar Island (2015), and the totality of the material collected in Ollagüe (2012), Caleta Vitor and Mocha Island (2015) was preserved in 90 - 95% ethanol before been sent to the laboratory, all other ticks were brought alive. Upon arrival, part of the living females was separated and placed inside an incubator at 25°C and 80 – 85% of relative humidity in order to obtain unfed larvae. As some Chilean soft ticks lack a description of adult stages, a morphological identification was attempted using laboratory-reared larvae. On the other hand, for the remaining part of females, and other collected specimens, a tip of one leg was cut for DNA extraction, and the rest of the tick was frozen at -80 °C to perform further *Rickettsia* isolations. The prospections made inside Chilean National Parks and Reserves were authorized by the “Corporación Nacional” (CONAF) resolution 033/2014.

Figure 1. Map of Northern and Central Chile. Visited localities are marked with a black dot. Dotted lines within Chile delimit the administrative regions of the country.

4.2.2. Morphological identification of ticks

One week after eclosion, ten unfed larvae per individual female offspring were separated and killed in hot water to perform a morphological diagnosis. Larvae were clarified in a 20% KOH solution, and mounted in slides using Hoyer's medium. Morphological characters were observed through light microscopy (Olympus BX40 optical microscope) and compared with published descriptions of Chilean (Kohls and Hoogstraal, 1961; Clifford et al., 1980; Hoogstraal et al., 1985; Estrada-Peña et al., 2003; Venzal et al., 2012, 2015, 2013, 2008; Muñoz-Leal et al., 2016) and other Neotropical soft ticks (Jones and Clifford 1972, Keirans et al. 1979). Ethanol preserved adult stages were compared with original descriptions of other Neotropical Argasidae, if available (Clifford et al., 1980; Hoogstraal et al., 1985; Estrada-Peña et al., 2003; Venzal et al., 2008; Muñoz-Leal et al., 2016), and nymphs were exclusively identified by molecular tools.

4.2.3. Molecular tools

To confirm morphological diagnoses, DNA extraction using the Guanidine Isothiocyanate technique (Sangioni et al. 2005) was individually performed to 27 laboratory-reared larvae, to the leg tips of frozen adults and nymphs, and to ethanol-preserved ticks in order to amplify a $\approx$ 460-bp fragment of the tick mitochondrial 16S rRNA gene by conventional PCR (Mangold et al. 1998). If negative by this PCR assay, samples were discarded and excluded from further analyses. To detect rickettsial DNA, each positive extracted tick was submitted to an initial screening using primers CS-78 and CS-323, targeting a conserved fragment of the citrate synthase gene (*gltA*) that occurs in all *Rickettsia* species (Labruna et al. 2004). Samples yielding expected size amplicons were subsequently submitted to different PCR protocols using primers targeting partial fragments of the rickettsial genes *gltA*, *ompA*, *ompB* and *htrA* (Table 1). All positive PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, USA) and further sequenced using the BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Austin, USA) in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, ABI 3500 Genetic Analyzer, Foster City, CA) with the same primers selected for PCR. Generated sequences were assembled, primer-trimmed, and translated to amino

acid (if applicable) with Geneious R9 software (Kearse et al. 2012), and then submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) in order to infer closest similarities with other published organisms available in GenBank (Altschul et al. 1990). Sequences not codifying a clean succession of amino acids after translation were discarded.

Table 1. Targeted genes, list of primers and PCR protocols used in this study.

Gene	Function	Primer	Nucleotide sequence (3'-5')	PCR protocol/Fragment size	Reference
Tick					
16S rRNA	Mitochondrial major ribosomal subunit	16S+1F 16S-1R	CCGGTCTGAACTCAGATCAAGT GCTCAATGATTTTTTAAATTGCTGT	PCR: 16S+1/16S-1: 460 pb	Mangold et al. (1998)
<i>Rickettsia</i>					
<i>gltA</i>	Citrate Synthase protein	CS78F CS323R CS239F CS1069R	GCAAGTATCGGTGAGGATGTAAT GCTTCCTTAAAATTCAATAAATCAGGAT GCTCTTCTCATCCTATGGCTATTAT CAGGGTCTTCGTGCATTCTT	PCR: CS78F/CS323R: 401 pb PCR: CS239F/ CS1069R: 830 pb	Labruna et al. (2004)
<i>ompA</i>	Outer membrane protein A	190.70F1 190.701R2 190.602R1	ATGGCGAATATTTCTCCAAAA GTTCCGTTAATGGCAGCATCT AGTGCAGCATTCGCTCCCCCT	1stPCR: 190.70F1/190.701R2: 617 pb Hemi-nested: 190.70F1/190.602R1: 512 pb	Regnery et al. (1991)
<i>ompB</i>	Outer membrane protein B	190.59F 190.807R	CCGCAGGGTTGGTAACTGC CCTTTTAGATTACCGCCTAA	PCR 190.59F/190.807R: 862 pb	Roux and Raoult (2000)
<i>htrA</i>	17 kDa protein	17K3F 17K4R 17K1F 17K2R	GCTCTTGCAACTTCTATGTT CATTGTTTCGTCAGGTTGGCG GCTTTACAAAATTCTAAAAACCATATA TGTCTATCAATTCACAACTTGCC	1stPCR: 17K3/17K4: 549 pb Nested: 17K1/17K2: 439 pb	Webb et al. (1990)

4.2.4. Isolation of *Rickettsia* spp..

Attempts to isolate rickettsiae in cell culture were performed with eleven ticks (four collected in 2014, three in 2015, and four in 2016). For this purpose, each tick was thawed and immediately subjected to the shell vial technique for isolation of rickettsiae in Vero cell culture, as previously described (Labruna et al. 2004). Briefly, cultures of Vero cells were inoculated with tick-body homogenates and incubated at 28° C. Every three days, the medium was switched to new medium without antibiotics and antifungal, and the aspirated medium was checked for the presence of *Rickettsia*-like organisms. The percentage of Vero cells infected with rickettsiae was monitored by the use of Giménez staining of cells scraped from each inoculated monolayer. After the establishment of each isolate in the laboratory (i. e., at least three cell passages with >90% infected cells), rickettsial DNA was extracted from the infected cells using the DNeasy® Blood and Tissue Kit (QIAGEN®) (Labruna et al. 2004). The extracted DNA was tested with primer pairs listed in Table 1. From each of the eleven shell vial attempts, part of the tick homogenate was also tested by PCR targeting the *gltA* gene. All expected size-PCR products were purified, sequenced and submitted to BLAST analyses as stated above.

4.2.5. Phylogenetic analyses

Individual alignments for each gene using sequences obtained in the current study, and from other *Rickettsia* species retrieved from GenBank (Table 2), were initially constructed with the CLUSTAL W algorithm (Thompson et al. 1994) implemented in Geneious R9 (Kearse et al. 2012). Subsequently, the alignments of *gltA*, *htrA*, *ompA*, and *ompB* were manually concatenated into the following three matrixes: *gltA-ompA-ompB-htrA*, *gltA-ompB-htrA*, and *gltA-ompB*. Taking into account the sequences of rickettsial genes obtained in the current study, and the available data in GenBank, the previous combinations were selected in order to maximize the quantity of rickettsial genes, and *Rickettsia* species included in each phylogenetic analysis. Each concatenated matrix was submitted to a Maximum Parsimony (MP) analysis using the software MEGA 5 (Tamura et al. 2011). Trees were obtained using the Tree-Bisection-Regrafting (TBR) algorithm, and 1000 bootstrap replications. Sequences of *Rickettsia canadensis* (U59713; CP000409;

EF160122; AF445381) were included as out-group in the *gltA-ompB-ompA-htrA* tree, and *Rickettsia bellii* (NC007940; CP000087; CP000087) rooted the *gltA-ompB-htrA*, and *gltA-ompB* trees.

Table 2. List of sequences retrieved from GenBank for the phylogenetic analyses.

Species	<i>gltA</i>	<i>ompA</i>	<i>ompB</i>	<i>htrA</i>
<i>Ca. Rickettsia andeanae</i>	GU169051	KF030932	GU395297	GU395295
<i>Ca. Rickettsia nicoyana</i>	KX228143	KX228144	KX228145	KX228146
<i>Ca. Rickettsia wissemannii</i>	LT558852	-	LT558854	-
<i>Ca. Rickettsia senegalensis</i>	KF666472	-	KT304219	KU167052
<i>Rickettsia aeschlimannii</i>	AY259084	DQ379982	AF123705	KT318742
<i>Rickettsia africae</i>	U59733	U43790	AF123720	KF646137
<i>Rickettsia akari</i>	NC009881	L01461	AF123707	KC588968
<i>Rickettsia amblyommatis</i> AL	EU274654	EU274656	EU274657	EU274655
<i>Rickettsia amblyommatis</i> ARANHA	AY360216	AY360213	AY360214	AY360215
<i>Rickettsia amblyommatis</i>	JX867425	KY628367	KX151487	KJ534311
<i>Rickettsia asembonensis</i>	JN315968	JN315977	JN315972	JN315975
<i>Rickettsia australis</i>	U59718	AF149108	AF123709	M74042
<i>Rickettsia bellii</i>	-	NC007940	CP000087	CP000087
<i>Rickettsia buchneri</i>	JFKF01000076	-	JFKF01000185	KDO03625
<i>Rickettsia canadensis</i>	U59713	EF160122	CP000409	AF445381
<i>Rickettsia conorii</i>	AE006914	AY346453	AE006914	JN182795
<i>Rickettsia felis</i>	JQ674484	DQ408668	AF182279	GU447234
<i>Rickettsia heilongjiangensis</i>	CP002912	AF179362	CP002912	EU503184
<i>Rickettsia helvetica</i>	KF859959	-	AF123725	GU827073
<i>Rickettsia honei</i>	AF018074	DQ309096	AF123711	AF027124
<i>Rickettsia hoogstraalii</i>	CCXM01000001	EF629537	CCXM01000001	FJ767736
<i>Rickettsia japonica</i>	U59724	U43795	AF123713	DQ909071
<i>Rickettsia lusitaniae</i> CEVDI PoTiRo	JQ771933	JQ771935	JQ771933	JQ771934
<i>Rickettsia lusitaniae</i> RLOYUCMEX001	KX377431	-	KX377433	-
<i>Rickettsia massiliae</i>	U59719	DQ212707	AF123714	JN871835
<i>Rickettsia monacensis</i>	DQ100163	AH015165	EF380356	EF380355
<i>Rickettsia montanensis</i>	U74756	FM883670	AF123716	DQ402377
<i>Rickettsia parkeri</i>	U59732	KY124258	AF123717	KF782322
<i>Rickettsia peacockii</i>	CP001227	FM883671	CP001227	CP001227
<i>Rickettsia prowazekii</i>	U59715	-	CP004889	AY730679
<i>Rickettsia raoultii</i>	EU036985	AH015610	EU036984	KT261761
<i>Rickettsia rickettsii</i>	CP000766	KF742603	GU395293	GU723477
<i>Rickettsia rhipicephali</i>	DQ865206	KX099901	DQ865209	U11020
<i>Rickettsia sibirica</i>	U59734	U43807	AY331393	AF445384
<i>Rickettsia slovaca</i>	U59725	U43808	AF123723	JN182788
<i>Rickettsia</i> sp. Atlantic rainforest	GQ855235	KJ855085	GQ855236	KJ855084
<i>Rickettsia tamurae</i>	AF394896	DQ103259	DQ113910	KJ855084
<i>Rickettsia tasmanensis</i>	GQ223391	-	GQ223393	-
<i>Rickettsia typhi</i>	NC006142	-	NC006142	KF241855
<i>Rickettsia vini</i>	KJ626334	JF758828	KT187395	JF758827

4.3. RESULTS

4.3.1. Ticks

A total of 239 ticks (39 larvae, 76 nymphs, 60 females and 54 males) were collected in the different visited localities (Table 3). With the exception of females collected in Pampa del Tamarugal National Reserve and Nevados de Chillán, fertile females from Santa María Island, Calama, Taltal and Pan de Azúcar National Park laid eggs in the laboratory. By the morphological study of larval stages, four species of *Ornithodoros* and two of *Argas* were identified as follows: 1) *A. cucumerinus*: dorsal plate suboval, irregular in shape, dorsum with 61 – 72 setae, posthypostomal setae ph1 markedly larger than ph2, tarsus I with five pairs plus one single seta ventrally and two pairs laterally; Haller's organ with coralline reticulations and with a trumpet-shape sensilla projecting posteriorly into the lumen of tarsus I (Clifford et al. 1978) (Figure 2A, C, E, G, J and K); 2) *Argas persicus* (Oken 1818): dorsal plate subcircular, tarsus I with one pair of lateral and two pairs of ventral setae, dorsum with 14 – 16 dorsolateral setal pairs, posterolateral and anterolateral setae nearly equal in length (≈ 0.039 mm), Haller's organ without coralline reticulations and lacking a trumpet-like sensillum (Kohls et al. 1970) (Figure 2B, D, F, H, and I); 3) *Ornithodoros atacamensis* Muñoz-Leal, Venzal & González-Acuña 2016: dorsal plate large and pyriform in shape, dorsum provided with 17 pairs of setae, 10 dorsolateral and five central, basis capituli with posterior margin slightly curved, hypostome arising from the basis on a median extension, pointed apically, dental formula 3/3 in the anterior third, then 2/2 towards the base, file one with 19 – 22 denticles, file 2 with 18 – 21 and file 3 with 10 – 12 (Muñoz-Leal et al. 2016) (Figure 3A, D and F); 4) *Ornithodoros microlophi* Venzal, Nava & González-Acuña 2013: dorsal plate pyriform, dorsum provided with 19 – 20 pairs of setae, 13 – 14 dorsolateral 6 – 8 central, 6 pairs of sternal setae, posterior margin of basis capituli straight, hypostome apically pointed, dental formula 2/2 – 3/3 apically, 4/4 in median portion then 2/2 towards de base, denticles of files variable in number, file one with 24 – 25 denticles, file 2 with 22 – 25, file 3 with 16 – 18 and file four with 1 -12 (Venzal et al. 2013) (Figure 3B, C, E, and G); 5) *Ornithodoros peruvianus* Kohls, Clifford & Jones 1969: dorsum with 14 pairs of setae, 11 dorsolateral and three central, posterior margin of basis capituli broadly rounded, hypostome arising from a median extension, tapering

abruptly to a blunt apex, dental formula 3/3 in the anterior two-third, then 2/2 towards the base, file one with 18 denticles, two with 17, and 3 with 13, tarsus I with barbed setae (Kohls et al. 1969) (Figure 4A, C, D, and F); and, 6) *Ornithodoros rioplatensis*: dorsum provided with 19 – 20 pairs of setae, 15 dorsolateral and five central, dorsal plate pyriform, hypostome dental formula with three files in the distal third and two towards the base; file one with 22-24 denticles, two with 21-22, and three with 11-12 (Figure 4B, E, and G). Vouchers of slide-mounted larvae were deposited at the “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (CNC) under the following numbers: CNC-3298, -3299, -3589, -3590, -3591, and -3593.

In addition, adult stages of *Argas neghmei* Kohls & Hoogstraal 1961 were recognized by the following combination of characters: lateral integument divided by a continuous suture; dorsolateral surface provided with *persicus*-like cells separated by a group of *reflexus*-like striations, and presence of one subapical dorsal protuberance in tarsi I (Kohls and Hoogstraal 1961). Adults of *Ornithodoros spheniscus* Hoogstraal, Wassef, Hays and Keirans 1985, collected in Pan de Azúcar National Park and The Dead Islet were differentiated from other congeneric seabird ticks by possessing an anteromedian disc in the anterior part of the dorsum. Finally, adults, nymphs and larvae of an *Ornithodoros* species, morphologically closely related to *Ornithodoros rioplatensis* Venzal, Estrada-Peña & Mangold 2008, were collected in Nevados de Chillán. Allotments for nymphal and mature stages are as follow: CNC-3594, -3595, -3596, -3597, and -3599.

Figure 2. Micrographs of *Argas* cf. *A. cucumerinus*, and *A. persicus* larvae. *Argas* cf. *A. cucumerinus*: (A) dorsal view, (C) hypostome, (E) ventral posterior view, (G) dorsal plate, (J) reticulations of Haller's organ surface and trumpet-like sensilla (arrowed), and (K) five ventral pairs of tarsus I (arrowed). *Argas persicus*: (B) dorsal view, (D) hypostome, (F) ventral posterior view, (H) Haller's organ, and (I) dorsal plate. Scale is given in millimeters.

Figure 3. Micrographs of *O. atacamensis* and *O. microlophi*. *Ornithodoros atacamensis*: (A) dorsal view, (D) hypostome, and (F) dorsal plate. *Ornithodoros microlophi*: (B) dorsal view, (C) ventral view, (E) hypostome, and (G) dorsal plate. Scale bar is given in millimeters. Abbreviations: Al, anterolateral setae, C, central setae, St, sternal setae, and Ca, circumanal setae.

Figure 4. Micrographs of *O. peruvianus* and *O. cf. rioplatensis* larvae. *Ornithodoros peruvianus*: (A) dorsal view, (C) tarsus I with barbed setae arrowed, (D) hypostome, and (F) dorsal plate. *Ornithodoros cf. O. rioplatensis*: (B) dorsal view, (E) hypostome, and (G) dorsal plate. Scale bar is given in millimeters. Abbreviations: Al, anterolateral setae, and C, central setae.

4.3.2. Molecular tools

4.3.2.1. Ticks

Molecular tools confirmed morphological diagnoses for larvae and adult specimens, and also for collected nymphs, since for each group of ticks identified to species level, sequencing of mitochondrial 16S rRNA yielded 99 – 100% identical sequences (data not shown). In addition, BLAST analyses of obtained sequences confirmed our morphological diagnoses, since the six analyzed tick species yielded mitochondrial 16S rDNA sequences showing 98 - 100% similarity with conspecific species sequences available in GenBank as well. Adults and nymphs of *Argas* collected in Ollaguë yielded identical sequences between them and 98% (405/414-pb) identical to *A. neghmei* (FJ853598). With the exception of two nymphs, one male and one female of *O. microlophi* collected in RNPT that yielded four different haplotypes for the 16S rDNA sequences, one haplotype per locality was retrieved for all other tick species. Noteworthy, after BLAST comparisons, sequences of the *Argas* species from Caleta Vitor were 99.7% (413/414-pb) identical with *A. neghmei* (FJ853598). However, morphology of adults, nymphs and larvae strikingly differed with *A. neghmei*, and matched the description of *A. cucumerinus* (Clifford et al. 1978). Consequently, following the syntaxis for a *conferre* (cf.) species (Lucas 1986), it was opted to treat the identified specimens as *Argas* cf. *A. cucumerinus*. In the same way, treatment as *Ornithodoros* cf. *O. rioplatensis* was adopted for the ticks collected in Nevados de Chillán, since although morphology of adults, nymphs and larvae was almost identical to *O. rioplatensis*, the identity of partial mitochondrial 16S RNA sequences between them was lower than 93% (398/420-pb).

4.3.2.2. *Rickettsia*

Overall, DNA of *Rickettsia* was amplified from each of the eight analyzed tick species. While almost all infected ticks yielded fragments of *gltA*, *ompB* and *htrA* genes, the *ompA* gene was only amplified from *Argas* cf. *A. cucumerinus*, *A. persicus*, *O. atacamensis*, *O. microlophi*, *Ornithodoros* cf. *O. rioplatensis*, and *O. peruvianus*. However, clean sequences were obtained only for *Argas* cf. *A. cucumerinus*, *O. atacamensis* and *O. microlophi* (Table 3). Only the *gltA* gene was

amplified from *A. neghmei* and *O. spheniscus*. All tick species showed *Rickettsia* prevalence higher than 70%, with the exception of *Argas* cf. *A. cucumerinus*, *O. atacamensis*, and *O. spheniscus*. Percentages of identity between rickettsial sequences obtained in this study with other known species are showed in Table 3. Remarkably, in some species of ticks all post-embryonic stages were positive to *Rickettsia* detection.

Table 3. Species and collection localities of analyzed soft ticks, prevalence of *Rickettsia* detection, and results of BLAST comparisons for each rickettsial gene (*gltA*, *ompA*, *ompB* and *htrA*). The identity of the first 100% cover query match is indicated. L: larvae; N: nymphs; M: males; F; females.

Tick species Locality (No. analysed specimens)*	Prevalence (No. positive ticks)	<i>gltA</i>	<i>ompA</i>	<i>ompB</i>	<i>htrA</i>
<i>Argas cf. A. cucumerinus</i> Caleta Vitor (3L, 15N, 1F)	26.6% (4N)	100% (776-bp) <i>Rickettsia lusitaniae</i> (JQ771933)	100% (488-bp) <i>Rickettsia lusitaniae</i> (JQ771935)	99.4% (795/800-bp) <i>Rickettsia lusitaniae</i> (JQ771937)	100% (394-bp) <i>Rickettsia lusitaniae</i> (JQ771934)
<i>Argas neghmei</i> Ollagüe (4N, 3F, 2M)	87.5% (3N, 3F, 2M)	98.4% (1088/1106-bp) <i>Rickettsia montanensis</i> (CP003340)	_____	_____	_____
<i>Argas persicus</i> Calama (9L, 9N, 13F, 5M)	96.6% (9L, 8N, 13F, 5M)	99.7% (1103/1106-bp) <i>Rickettsia hoogstraalii</i> (DQ081187)	_____	99.3% (812/818-bp) <i>Rickettsia hoogstraalii</i> (EF629536)	100% (393-bp) <i>Rickettsia hoogstraalii</i> (FJ767736)
<i>Ornithodoros atacamensis</i> Pan de Azúcar National Park (9L, 22N, 5F, 6M)	5.3% (1N, 1M)	99.2% (1096/1105-bp) <i>Rickettsia amblyommatis</i> (CP012420)	98.5% (578/587-bp) <i>Rickettsia amblyommatis</i> (CP012420)	97.7% (797/816-bp) <i>Rickettsia amblyommatis</i> (CP012420)	99.6% (456/458-bp) <i>Rickettsia amblyommatis</i> (KJ534311)
<i>Ornithodoros microlophi</i> Santa María Island (9L, 2N, 2F, 5M)	91.7% (9L, 1N, 2F, 5M)	97.8% (1082/1106-bp) <i>Rickettsia montanensis</i> (CP003340)	98.6% (481/488-bp) <i>Rickettsia amblyommatis</i> (CP015012)	91.5% (748/817-bp) <i>Rickettsia tasmanensis</i> (GQ223393)	98.0% (386/394-bp) <i>Rickettsia</i> sp. ChinaDs61 (AB795131)
Pampa del Tamarugal National Reserve (7N, 1M, 1H)	77.7% (1N, 6F)	98.0% (1039/1062-bp) <i>Rickettsia montanensis</i> (CP003340)	98.0% (478/488-bp) "Candidatus <i>Rickettsia andeanae</i> " (KY628370)	91.7% (742/809-bp) <i>Rickettsia tasmanensis</i> (GQ223393)	98.0% (488/499-bp) <i>Rickettsia raoultii</i> (CP019435)
<i>Ornithodoros peruvianus</i> Taltal (5L, 16N, 2F, 8M)	74.1% (5L, 9N, 2F, 7M)	99.9% (775/776-bp) <i>Rickettsia lusitaniae</i> (JQ771933)	_____	98.6% (812/823-bp) <i>Rickettsia lusitaniae</i> (JQ771937)	100% (394-bp) <i>Rickettsia lusitaniae</i> (JQ771934)
Pan de Azúcar Island (5F, 4M)	77.7% (4F, 2M)	idem	_____	idem	idem
<i>Ornithodoros spheniscus</i> Pan de Azúcar Island (10M, 10F)	10% (1M, 1F)	_____	_____	_____	_____
The Dead Islet, Mocha Island (11M, 11F)	13.6% (3F)	100% (350-bp) <i>Rickettsia felis</i> (CP000053)	_____	_____	_____
<i>Ornithodoros cf. O. rioplatensis</i> Nevados de Chillán (4L, 1N, 2M, 7F)	92.9% (4L, 1N, 2M, 6F)	97.6% (1079/1106-bp) <i>Rickettsia montanensis</i> (CP003340)	_____	91.4% (752/823-bp) <i>Rickettsia raoultii</i> (CP010969)	98.0% (490/499-bp) <i>Rickettsia raoultii</i> (CP019435)

4.3.3. *Rickettsia* isolations

One female and one nymph of *A. persicus*, one male and one nymph of *O. peruvianus* (both collected in Pan de Azúcar National Park), two nymphs and one male of *O. atacamensis*, two nymphs of *O. microlophi* (collected in RNPT), and two nymphs of *Ornithodoros* cf. *O. rioplatensis* were submitted to the Shell Vial technique. Although rickettsial DNA was amplified in each of the 11 individually processed ticks, successful isolations were obtained only from one nymph of *A. persicus*, one nymph and one male of *O. atacamensis*, one nymph of *O. microlophi*, and one nymph of *O. peruvianus*. Sequences of genes obtained from the isolated rickettsiae shared 100% identity with the rickettsial genes characterized directly from tick-extracted DNA in these three tick species. Rickettsial isolates obtained in the present study have been deposited in the Rickettsial Collection of the Laboratory of Parasitic Diseases of the Faculty of Veterinary Medicine at the University of São Paulo.

4.3.4. Phylogenetic analyses

Overall, the topologies of the three phylogenetic reconstructions were very similar (Figure 5, 6, and 7), however the highest node bootstrap was observed in the *gltA-ompA-ompB-htrA* tree (Figure 3). In particular, there was a strong support for a monophyletic group of *Rickettsia* between the Spotted Fever Group (SFG) and the Transitional Group (TRG), composed by the organisms detected in *O. microlophi* and *Ornithodoros* cf. *O. rioplatensis* (Figure 5, 6, and 7). The phylogenetic positions of the rickettsiae characterized from *O. atacamensis*, and *Argas* cf. *A. cucumerinus* were also stable in all three trees, and supported genetic identities with *R. amblyommatis* and *R. lusitaniae*, respectively. Supported only by the *gltA-ompB-htrA* and *gltA-ompB* trees, genetic identities of the rickettsiae characterized from *O. peruvianus* and *A. persicus*, clustered into monophyletic groups with *R. lusitaniae* and *R. hoogstraalii*, respectively. The following names are proposed to the *Rickettsia* strains characterized at least for *gltA*, *ompB* and *htrA* genes: *R. amblyommatis* Atacama strain (isolated from *O. atacamensis*), *R. hoogstraalii* Calama strain (isolated from *A. persicus*), *R. lusitaniae* OPeChile strain (isolated from *O. peruvianus*), *R. lusitaniae* ACuChile strain (characterized from *Argas* cf. *A. cucumerinus*), *Rickettsia* sp.

OMiSMiChile strain (characterized from *O. microlophi* collected in Santa María Island), *Rickettsia* sp. OMiTamChile strain (isolated from *O. microlophi* collected in Pampa del Tamarugal National Reserve), and *Rickettsia* sp. Shangrilá strain (characterized from *Ornithodoros* cf. *O. rioplatensis*).

Figure 5. MP phylogenetic tree inferred upon the concatenated *gltA*, *ompA*, *ompB* and *htrA* genes of 35 species of *Rickettsia*. A violet and light blue background denotes agents from spotted fever group and transitional group, respectively. Green background highlights the monophyletic group composed by *Rickettsia* spp. from *O. microlophi*. A gray background denotes the rooting groups. *Rickettsia* species characterized in the current study are highlighted in red bold. Nodes with bootstrap values lower than 0.70 are not shown.

Figure 6. MP phylogenetic tree inferred upon the concatenated *gltA*, *ompB* and *htrA* genes of 44 species of *Rickettsia*. A violet and light blue background denotes agents from spotted fever group and transitional group, respectively. The Typhus Group is placed over light orange, and a green background highlights the monophyletic group composed by *Rickettsia* spp. from *O. microlophi* and *Ornithodoros* cf. *O. rioplatensis*. A gray background denotes the rooting groups. *Rickettsia* species characterized in the current study are highlighted in red bold. Nodes with bootstrap values lower than 0.70 are not shown. Abbreviation: Ca., *Candidatus*.

Figure 7. MP phylogenetic tree inferred upon the concatenated *gltA* and *ompB* genes of 48 *Rickettsia* organisms. The violet and light blue backgrounds denote agents from spotted fever group and transitional group, respectively. The Typhus Group is placed over light orange, and a green background highlights the monophyletic group composed by *Rickettsia* spp. from *O. microlophi* and *Ornithodoros* cf. *O. rioplatensis*. A gray background denotes the rooting groups. *Rickettsia* species detected or isolated from soft ticks are in red (in red bold the organisms characterized in the current study). Nodes with bootstrap values lower than 0.70 are not shown. Abbreviations: *Ca*, *Candidatus*; *O.*, *Ornithodoros*; *Ot.*, *Otobius*.

4.4. DISCUSSION

4.4.1. Ticks

All ticks collected in the current study were identified either by a morphological or a molecular analysis. This twofold approach was conclusive for determining the identities of *A. persicus*, *A. neghmei*, *O. atacamensis*, *O. microlophi*, *O. peruvianus*, and *O. spheniscus*. However, morphological and genetic divergences were inconclusive in two cases. Although morphology of larvae and adult *Argas* ticks collected in Caleta Vitor matched the original description of *A. cucumerinus* (Clifford et al. 1978), obtained mitochondrial 16S rDNA sequences were almost identical to *A. neghmei* (99.7%, 413/414-pb). A similar genetic scenario between two *Argas* species has been pointed by Burger et al. (2014) and Muñoz-Leal et al. (2017) affecting *Argas miniatus* Kock 1844 and *Argas robertsi* Hoogstraal, Kaiser & Kohls, 1968. Conversely, a considerable genetic divergence (7.4%) suggestive of two separated soft tick lineages is not reflected in morphological traits of *Ornithodoros* ticks collected in Nevados de Chillán, since they are indistinguishable from *O. rioplatensis*. Remarkably, *O. rioplatensis* and the Chilean form possess geographical distribution abruptly separated by the Andes Mountains, which could correspond to an effective barrier defining the occurrence of two sibling taxa adapted to parasitize ecologically similar hosts. With the current genetic and morphological evidence, both above exposed taxonomic problems are not conclusively solved, therefore further analyses including the characterization of more genetic markers, and crosses between different *Argas* and *Ornithodoros* populations are needed in order to elucidate these systematic conflicts.

4.4.2. Rickettsiae and soft ticks

Although during the last decade the knowledge on soft tick-associated rickettsiae has increased, it is still limited, and many gaps preclude a reasonable comprehension of their eco-epidemiology. In terms of diversity of infected species and their respective ecological niches, the geographical distribution of *Rickettsia*-infected soft ticks resembles a map with “blinking” reports scattered in the American continent (Philip et al., 1983; Reeves et al., 2006; Mattila et al., 2007; Sánchez-

Montes et al., 2016; Tahir et al., 2016; Moreira-Soto et al., 2017;), Africa (Pader et al., 2012; Dietrich et al., 2014a; Lafri et al., 2015; Duron et al., 2017), Europe (Reháček et al., 1977; Milhano et al., 2014; Dupraz et al., 2017;), and northwestern Asian islands (Kawabata et al. 2006). Remarkably, some *Rickettsia* spp. naturally infect representatives of the Argasidae and Ixodidae families, which are the cases of *R. bellii* (Philip et al. 1983), *R. hoogstraalii* (Duh et al. 2010), *R. rickettsii* (Silva-Goytia and Elizondo 1952), and the herein find of a *R. amblyommatis* strain in an *Ornithodoros* tick. In soft ticks, all these species and strains lack a proper demonstration of perpetuation routes using vertebrate hosts as part of their natural cycles (i. e. horizontal transmission), and, with the exception of *R. rickettsii*, are still classified as organisms of uncertain pathogenicity (Duh et al., 2010; Milhano et al., 2014; Ereemeeva and Dasch, 2015). Hypothetically, horizontal transmission would imply that ticks infected with a same *Rickettsia* strain had to have common feeding preferences for one or a group of vertebrate hosts within a particular area. Although plausible, this assumption needs further research, since *R. hoogstraalii* lacks data on transmission pathways, and neither *R. amblyommatis* or *R. bellii* have a clearly demonstrated capacity to infect vertebrate tissues (Ereemeeva and Dasch 2015).

In contrast, suggestive data on transtadial perpetuation (i. e. vertical transmission) has been evidenced upon the detection of *Rickettsia*-positive embryonic, and postembryonic stages in several soft tick species, and was confirmed by the results for some rickettsial agents detected in this study. *Rickettsia lusitaniae* was detected in females, males and nymphs of *Ornithodoros erraticus* Lucas 1849 (Milhano et al. 2014), and *Ornithodoros yumatensis* Cooley & Kohls 1941 (Sánchez-Montes et al. 2016); and, all postembryonic stages of *O. peruvianus* were positive to this *Rickettsia* sp. in this study (Table 3). In turn, *R. hoogstraalii*-infected embryonic cells from *Ornithodoros capensis* Neumann 1901 were the first source of isolation for this rickettsial organism (Mattila et al. 2007). Moreover, in this study all postembryonic stages of *A. persicus* from Chile were positive to this rickettsia species (Table 3). Overall, high prevalence of *R. lusitaniae*-infected stages from *O. peruvianus*, and *R. hoogstraalii*-infected stages from *A. persicus*, suggest the occurrence of transtadial perpetuation for both *Rickettsia* sp. in both tick species, respectively. This situation was also observed for postembryonic stages of *A. neghmei*, *O. microlophi* and *Ornithodoros* cf. *O. rioplatensis* (Table 3). Conversely, the low rickettsial prevalence noticed for screened stages of *Argas* cf. *A.*

cucumerinus, *O. atacamensis* and *O. spheniscus*, suggests at least, that their associated rickettsiae would be anyhow less successful to maintain their populations by transovarian transmission.

4.4.2.1. *Rickettsia amblyommatis*

The isolation of a *R. amblyommatis* strain from *O. atacamensis* is intriguing. To date, this rickettsia was only known to infect several Amblyomminae species (Karpathy et al. 2016), and also *Dermacentor nitens* Neumann, 1897 and *Rhipicephalus microplus* (Canestrini, 1887) (Parola et al. 2013) across the American continent. The current results constitute the first time that *R. amblyommatis* has been detected and isolated from an argasid tick. Experimental studies and field surveys have demonstrated that *R. amblyommatis* is transmitted to mammals, and induce seroconversion in rabbits (Saraiva et al. 2013), and exposed dogs (Costa et al. 2017). Such a capacity of infecting mammal hosts would eventually allow a horizontal transmission to other ticks. For instance, until such a mechanism of colonizing a new tick host is not properly rejected, it would constitute a plausible pathway in order to explain the occurrence of *R. amblyommatis* in *O. atacamensis*. However, the fact that *O. atacamensis* is a reptile-associated tick chiefly distributed in the Atacama Desert (Muñoz-Leal et al. 2016), and away from any other reported *R. amblyommatis*-infected tick (Parola et al., 2013; Karpathy et al., 2016), tames the current knowledge on natural tick hosts, and geographical distribution of this rickettsial agent.

4.4.2.2. *Rickettsia hoogstraalii*

Firstly isolated from cell lines of *O. capensis* in the United States (Mattila et al. 2007), *R. hoogstraalii* was subsequently detected in *O. capensis* from Indian Ocean Islands (Dietrich et al. 2014b). This evidence, in conjunct with previous sequences obtained from *O. capensis* and *Ornithodoros sawaii* Kitaoka & Suzuki 1973 from Japan (Kawabata et al. 2006), confirm to date that only two *Ornithodoros* species harbor this *Rickettsia*. In addition, *R. hoogstraalii* was subsequently also isolated from *Haemaphysalis sulcata* Canestrini & Fanazgo 1878 from Croatia (Duh et al. 2010), and molecularly characterized from *A. persicus* in Ethiopia as well (Pader et al. 2012). Thus, it is not surprising that this rickettsia also infects *A. persicus* from

Chile. However, the importance of this finding falls in that the agent was successfully isolated for the first time from an *Argas* representative, and confirms that *R. hoogstraalii* does infect at least one species of *Argas*, *Haemaphysalis* and *Ornithodoros* genus with vastly distanced distributions.

4.4.2.3. *Rickettsia lusitaniae*

Differing from *R. hoogstraalii*, to date, *R. lusitaniae* has been exclusively characterized from soft ticks. Its first isolation was from *O. erraticus* (Lucas 1849) in Portugal (Milhano et al. 2014), and subsequent reports include its isolation from *O. yumatensis* from Mexico (Sánchez-Montes et al. 2016). Yet, by comparing a partial fragment of *gltA* gene, a related strain has been also recently detected in *Ornithodoros maritimus* Vermeil & Marguet 1967 from France (Dupraz et al. 2017). Here, *O. peruvianus*, and *Argas* cf. *A. cucumerinus* from Chile are added as new hosts for this bacterium. Mirroring the scenario with *R. hoogstraalii*, ticks harboring *R. lusitaniae* infections have vastly separated distributions, however they share somewhat similar ecological features: *O. peruvianus* and *O. yumatensis* are bat-associated ticks, and *Argas* cf. *A. cucumerinus* and *O. maritimus* are seabird parasites. While horizontal transmission has still to be demonstrated for *R. lusitaniae*, infections harbored by ticks with common ecological niches correspond to an interesting point that needs further assessment.

4.4.2.4. *Rickettsia* sp. from *O. microlophi* and *Ornithodoros* cf. *O. rioplatensis*

The results of this study confirm for the first time that *O. microlophi* and an *Ornithodoros* species phenotypically closely related to *O. rioplatensis* maintain rickettsial infections in nature. Sequences of molecularly characterized rickettsiae form a monophyletic clade between the SFG and TRG (Figures 5, 6 and 7). This position as a separated group of rickettsiae is also supported by genetic divergences observed for partial fragments of *gltA*, *ompA* and *ompB* genes (Table 2), which all exceed the accepted percentages that delimit species within the genus (Raoult et al. 2005). The existence of this lineage of *Rickettsia* basally positioned with respect to the classic SFG rickettsiae deserves more attention, since the agent from *O. microlophi* grows in vertebrate-cell cultures, however its putative pathogenic nature is

still unstudied.

4.4.2.5. Other *Rickettsia* spp. detected in this study

Apart from molecularly confirm the presence of rickettsial DNA, the shortness of the *gltA* sequences obtained for *A. neghmei* and *O. spheniscus* precludes further specific conclusions. Noteworthy, *O. spheniscus* belongs to a group of seabird-associated soft ticks. Interestingly the sequence of *gltA* gene obtained from *O. spheniscus* was 100% (350-pb) identical to *Rickettsia felis*, a TRG rickettsia (Table 2). This identity matches published findings in other soft ticks, such as *Ornithodoros capensis* (Kawabata et al., 2006; Reeves et al., 2006; Mattila et al., 2007; Dietrich et al., 2014b), *Ornithodoros maritimus* (Dupraz et al. 2017), and *Ornithodoros sawaii* (Kawabata et al. 2006), in which the isolated or detected rickettsiae cluster all within the TRG.

4.4.2.6 Other *Rickettsia* spp. detected in soft ticks

Interestingly, the most pathogenic agent of SFG rickettsiae, *R. rickettsii*, has also been found in soft ticks during earlier studies (Silva-Goytia and Elizondo 1952), and experimental work has demonstrated that several species can effectively transmit the bacterial agent (Hoogstraal 1985). The strains of *R. rickettsii* isolated from naturally infected *O. nicolleti* and *O. lagophilus* in Mexico (Silva-Goytia and Elizondo 1952) are a good example of this possible natural association. Notwithstanding, both isolates lack an available voucher to perform a molecular characterization, which in addition to the uniqueness of the report, turns the performance of new isolations an imperative approach in order confirm these early findings. Likewise, the role of *O. nicolleti* and *O. lagophilus* as natural vectors of *R. rickettsii* must still be addressed. However, in a series of previous studies, transstadial perpetuation and transovarian transmission of *R. rickettsii* strains was already demonstrated for *O. nicolleti* (Davis 1943a), and for the closely related *Ornithodoros parkeri* Cooley, 1936 (Davis, 1943b). Yet both species failed to maintain the infection after several generations (Davis, 1943a, 1943b). Noteworthy, experimentally infected *Ornithodoros rudis* Karsch 1880 do not have the capacity for transmitting *R. rickettsii* strains, but can maintain the infection transtadially for at least 243 days, and pass it through the egg to the next

generation (Davis, 1943c)

Recently, two new agents phylogenetically related between each other, and also to *R. rickettsii*, namely *Ca. Rickettsia nicoyana* and *Ca. Rickettsia wissemanni*, have been isolated from the ticks *Ornithodoros knoxjonesi* Jones & Clifford, 1972 (Moreira-Soto et al. 2017), and *Ornithodoros hasei* (Schulze, 1935) (Tahir et al. 2016), respectively. While vertical transmission of both *Rickettsia* spp. was not assessed, the occurrence of two genetically related *Rickettsia* in two bat-associated soft ticks rather points the role of horizontal transmission as a possible pathway to host switch. Subsequently, a respective adaptation process would explain genetic divergences exhibited by both *Rickettsia* sp.

Currently, in GenBank there is a collection of soft tick-retrieved *Rickettsia* sequences corresponding to fragments of *gltA*, *ompA*, *ompB* and *htrA* genes. However, some of them were excluded from this study whether by not corresponding to the same gene fragments that were included in the phylogenetic analyses, or by the shortness of the sequences.

4.4.3. Identities and phylogenetic position of soft tick rickettsiae detected in this study

An accurate specific classification for the rickettsiae characterized in the current study needs further information. In addition to the four genes herein analyzed, sequences of the *rrs* and *sca4* genes were not amplified. As a comparison of *rrs*, *gltA*, *ompA*, *ompB* and *sca4* genes is necessary to perform a species level inference in the genus *Rickettsia* (Raoult et al. 2005), identities of the agents presented in the current study should be considered as closely related organisms with already validated species. Notwithstanding, taking into account that the obtained sequences of *gltA*, *ompA*, *ompB* and *htrA* genes fulfill the boundaries of identity proposed by Raoult et al. (2005), rickettsial organisms characterized from *Argas* cf. *A. cucumerinus* and *O. atacamensis*, could indeed correspond to strains of *Rickettsia lusitaniae* and *Rickettsia amblyommatis*, respectively. In this way, almost identical sequences for *gltA*, *ompB* and *htrA* genes retrieved from *A. persicus* and *O. peruvianus*, could also point the respective occurrence of *Rickettsia hoogstraalii*, and again, *R. lusitaniae* strains in both soft ticks species.

Phylogenies of genus *Rickettsia* have put in evidence that pathogenic and

endosymbiotic forms do not define monophyletic groups, and rather exhibit a scattered pattern of infection among their invertebrate hosts (Perlman et al. 2006, Weinert et al. 2009). This ecological scenario is also reflected in ticks (Duron et al. 2017), and was supported by all three phylogenetic analyses performed in the current study, since *Rickettsia* spp. associated to herein examined soft ticks clustered within separated major clades, either within the SFG or TRG. Although in this study new associations between five soft ticks and their harbored rickettsiae were revealed, a deeper understanding on the evolutionary history between these organisms still needs an extensive search for rickettsial in still non screened soft tick species.

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5. ANAPLASMATACEAE, *Borrelia*, AND *Hepatozoon* AGENTS ASSOCIATED TO NATIVE TICKS (ACARI: ARGASIDAE, IXODIDAE) FROM CHILE

5.1. INTRODUCTION

Within the South American fauna of native ticks, pathogenic microorganisms associated to species parasitizing wild animals have been less studied if compared with the representatives associated with domestic animals or human activities. Apart from the unsolved taxonomic status of several species (Guglielmone et al. 2009), the South American fauna of ticks (Argasidae, Ixodidae) is composed by near 210 species (Guglielmone et al. 2003, Nava, Beati, et al. 2014, Nava, Mastropaolo, et al. 2014, Krawczak et al. 2015, Apanaskevich and Bermúdez 2017, Muñoz-Leal et al. 2017), and although it represents almost one fourth of the worldwide diversity of the subclass Ixodoidea, only few South American species of ticks are currently implicated as effective (Brumpt 1922, Forlano et al. 2005, Labruna 2009, Costa et al. 2017) or possible vectors of pathogenic agents to animals (Nava et al. 2017).

Alpha-proteobacteria of the Anaplasmataceae family are small parasitic bacteria of eukaryotic cells that replicate inside vacuoles derived from the host cell membrane (Kersters et al. 2006). Hard ticks constitute main invertebrate vectors of this bacterial group, which are transmitted to vertebrate hosts via saliva during the tick meal (Futse et al. 2003), infecting blood cells of mammal species, including humans (Ismail et al. 2010). Based on molecular evidence, Dumler et al. (2001) reorganized this family of proteobacteria into the following four genera: *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*. In addition, a potential fifth genus, "*Candidatus Neoehrlichia*", has been molecularly characterized from several ticks of

the genus *Ixodes*, tissues of their associated hosts (Kawahara et al. 2004), and from blood of ill tick-bitten humans as well (Li et al. 2012, Grankvist et al. 2015).

Tick also constitute natural vectors of borrelial spirochetes of either the Lyme Disease Group (LDG), composed by the *Borrelia burgdorferi* sensu lato (s. l.) genospecies ecologically associated chiefly with the genus *Ixodes* (Kurtenbach et al. 2006), and the Relapsing Fever Group (RFG), which includes more than 20 species mainly associated to *Ornithodoros* soft ticks (Cutler 2015). While in 1995, detection of LDG borreliae DNA in seabird ticks (i. e. *Ixodes uriae* White 1852) confirmed the presence of this bacteria in insular territories of the Southern Hemisphere (Olsen et al. 1995, Gylfe et al. 2001), it was only in recent years that molecular evidence (Barbieri et al. 2013, Nava et al. 2014, Sebastian et al. 2016, Dall'Agnol et al. 2017), and the isolation of *Borrelia chilensis* (Ivanova et al. 2014), demonstrated that LDG spirochetes do occur in South America. Conversely, the knowledge of RFG borreliae in this region of the American continent dates from the first half of 20th century, when experimental research on soft tick-borne borreliae enabled the documentation of several RFG species (Brumpt 1922, Davis 1952). In addition to this evidence, only one recent study confirmed by molecular tools that an *Ornithodoros* sp. was harboring bacteria of this group in Bolivia (Parola et al. 2011).

The protozoan genus *Hepatozoon* uses invertebrates as definitive hosts, and ectothermic and homeothermic vertebrates as intermediate ones (Smith 1996). While most species of *Hepatozoon* have been described to undergo sexual development and sporogony in hard ticks, the role of soft ticks as putative definitive hosts of protozoa of this genus has been scarcely assessed (but see Bennett et al., 1992), and never confirmed by molecular tools. While in South America *Hepatozoon canis* has been widely demonstrated to infect domestic animals (O'Dwyer et al. 2001, Eiras et al. 2007, Spolidorio et al. 2009, Vargas-Hernandez et al. 2012), molecular evidence obtained from wild vertebrates and their associated ticks possibly involved in the cycle of this protozoan, has increasingly been published in last decade (Merino et al. 2009, Wolf et al. 2016, Soares et al. 2017), which suggests that the diversity of the *Hepatozoon* genus in the Neotropics might be by far underestimated.

Native ticks from Chile are currently composed by 12 species of Ixodidae and 11 of Argasidae (Abarca et al., 2012; González-Acuña and Guglielmone, 2005; Muñoz-Leal et al., 2016a; Muñoz-Leal, chapter 3 to be published; Venzal et al., 2012, 2015, 2013, 2008). While native hard ticks are exclusively represented by the genera

Amblyomma and *Ixodes*, the genera *Argas* and *Ornithodoros* compose native soft ticks. In this country, previous research in tick-borne microorganisms enabled the detection of *Rickettsia* spp. in all three native *Amblyomma* species (Abarca et al. 2012, 2013, Muñoz-Leal et al. 2016b, Ogrzewalska et al. 2016), and one species of *Ixodes* has been documented to be infected with a LGD *Borrelia* (Ivanova et al. 2014, Verdugo et al. 2017).

A molecular characterization of microorganisms from tick-extracted DNA, along with a further phylogenetic comparison with organisms of known pathogenicity, constitutes a useful tool in order to infer putative tick vector roles (Tijssen-Klasen et al. 2014). It also becomes an important first-step approach to understand the diversity of harbored agents in poorly studied tick fauna. The limited knowledge on microorganisms harbored by native ticks from Chile prompted the realization of this study.

5.2. MATERIAL AND METHODS

5.2.1. Sites of study and collection of ticks

Between November 2014 and February 2017, ten sites of Northern, Central and Southern Chile were visited, as follows: Caleta Vitor (18°45'S; 70°20'W, elevation 23 m), Pampa del Tamarugal National Reserve (20°26'S; 69°41'W, elevation 1000 m), Santa María Island (23°26'S; 70°36'W, elevation 2 m), Taltal (25°23'S; 70°30'W, elevation 9 m), Pan de Azúcar National Park (26°17'S; 70°38'W, elevation 35 m), Bosque de Fray Jorge National Park (30°33'S; 71°37'W; elevation 519 m), Las Chinchillas National Reserve (31°30'S; 71°06'W; elevation 551 m), Mocha Island (38°23'S; 73°55'W, elevation 35 m), The Dead Islet (Mocha Island, 38°25'S; 73°55'W, elevation 6 m), and Bahía Eugenia at Navarino Island (54°56'S; 67°18'W, elevation 7 m) (Figure 1). With the exception of hard ticks collected from rodents in Bosque de Fray Jorge National Park (BJFNP), Mocha Island (MI), and from a bird in Bahía Eugenia (BE), and soft ticks collected from reptiles in Reserva Nacional Pampa del Tamarugal (RNPT), Pan de Azúcar National Park (PANP), and Las Chinchillas National Reserve (LCNR), ticks analyzed in this study have been reported in previous studies (Muñoz Leal et al. chapter 2, chapter 3, to be published). The collection of ticks upon their vertebrate hosts followed the resolutions 6007/2014

and 4470/2016 given by the “Servicio Agrícola y Ganadero (SAG)”. Fieldwork in National Parks and Reserves was authorized by the licenses 033/2014 and 039/2016 given by the “Corporación Nacional Forestal (CONAF)”.

In addition, material from the “Colección de Zoología de la Facultad de Ciencias Veterinarias de la Universidad de Concepción (CZFCUdeC)”, Chillán, Chile, was also examined as follows: two larvae, and 13 nymphs of *Ixodes neuquenensis* Ringuelet 1947, collected on *Dromiciops gliroides* (Marshall 1978) (Microbiotheria: Microbiotheriidae) at Cascadas (41°04'S; 72°38'W, elevation 70 m), date of collection: November of 2008; five females of *Ixodes taglei* Kohls 1969 collected on *Pudu puda* (Molina, 1782) (Artiodactyla: Cervidae), three at Santa Juana (37°10'S; 72°56'W, elevation 58 m), and two at Los Angeles (37°27'S; 72°21'W, elevation 135 m), date of collection: September of 2008; and, *I. uriae*, 12 nymphs, 13 females and nine males collected in the surroundings of Gabriel González Videla Base (GGVB), Antarctic Peninsula (64°49'S; 62°51'W, elevation 1 m), near *Pygoscelis* spp. (Sphenisciformes: Spheniscidae) colonies, date of collection: January of 2012.

Figure 1. Map of Chile with the 14 locations(black dots) where ticks were collected.

5.2.2. Identification of ticks

Collected adult hard ticks were examined using an stereomicroscope (SteREO Discovery V12), separated by stage, and identified following the keys of Nava et al. (2017), and additional species descriptions (Keirans et al. 1976, Guglielmone et al. 2010). While the majority of soft ticks analyzed in the current study came from collections carried out in previous studies, in which the identification was

morphologically and molecularly confirmed (Muñoz-Leal et al., chapter 2, and chapter 3, to be published), two herein collected larvae from Reserva Nacional Pampa del Tamarugal, five from Las Chinchillas National Reserve, and three from Pan de Azúcar National Park, were mounted in slides using Hoyer's medium to perform a morphological identification. Characters of larvae were observed through optical microscopy (Olympus BX40 microscope) following the key from Nava et al. (2017), and comparing with original descriptions of Chilean Ornithodorinae (Muñoz-Leal et al., 2016b; Muñoz-Leal, chapter 2 to be published). The identity of nymphs and hard tick-larvae, and of the material from CZFCUdeC was assessed exclusively by means of molecular tools.

5.2.3. Molecular tools

5.2.3.1. DNA extraction

Part of the specimens collected from vertebrates in the current study, and the material retrieved from the CZFCUdeC was submitted to DNA extraction. Briefly, in order to remove residuals of substrate, ticks were syringe-flushed with distilled water, and then submitted to three successive rinses with 70% ethanol and ultrapure water. Following this, the Guanidine Isothiocyanate technique (Sangioni et al. 2005) was played to each extracted sample. While the majority of larvae, nymphs and the totality of adult ticks were submitted to individual DNA extraction, part of hard tick larvae and nymphs collected from a same host were pooled. To molecularly identify some of the pooled ticks, one specimen per pool was separated for individual DNA extraction. The resulting pelleted DNA was eluted in 10 - 80 uL of TE (Tris-EDTA buffer solution).

5.2.3.2. PCR

A conventional PCR using primers 3'-CCGGTCTCAACTCAGATCAAGT-5' (forward) and 3'-GCTCAATGATTTTAAATTGCTGT-5' (reverse) targeting a ~460-bp fragment of the tick mitochondrial 16S rRNA gene (Mangold et al. 1998), was performed to confirm morphological diagnoses of larvae, nymphs and adults collected at BFJNP and MI, to larvae and nymphs collected in BE, larvae collected in

RNPT, PANP and RNLC, and ethanol-preserved specimens from the CZFCUdeC. Samples not yielding visible amplicons of the expected size in agarose gel were discarded and excluded from further analyses.

To test the presence of Anaplasmataceae, *Borrelia* and *Hepatozoon* DNA, different PCR screenings followed by nested reactions were performed in order to discriminate positive ticks. PCR protocols and primers are listed in Table 1. DNA of *Anaplasma platys*, *Ehrlichia canis*, *Borrelia anserina* and *Hepatozoon canis* were used as positive controls.

PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, USA) and further sequenced using the BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Austin, USA) in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyzer, Foster City, CA) with the same primers used for PCR. Obtained sequences were assembled, primer trimmed, and translated (if protein codifying) with Geneious R9 (Kearse et al. 2012). Nucleotide sequences yielding an imperfect succession of amino acids were discarded, and the rest was submitted to a BLAST analysis (www.ncbi.nlm.nih.gov/blast) to infer closest similarities with other known organisms (Altschul et al. 1990).

Tick-extracted DNA yielding Anaplasmataceae-positive sequences was subsequently submitted to a second round of PCR targeting genus specific genes *groESL*, *dsb* and *gltA* of *Anaplasma*, *Ehrlichia* and “*Ca. Neoerhlichia*” bacteria, respectively (Table 1). PCRs for “*Ca. Neoerhlichia*” were performed without positive controls. *Borrelia*-positive samples were submitted to a second PCR targeting a fragment of the *rrs* gene (Table 1).

Table 1. List of primers and PCR protocols used for the detection and characterization of tick microorganisms in the current study.

Organism/Gene	Function	Primer name	Sequence (5' to 3')	PCR/Amplicon Size	Reference
Anaplasmataceae					
<i>rrs</i>	Bacterial small ribosomal subunit	EHR16SD EHR16SR	GGTACCYACAGAAGAAGTCC TAGCACTCATCGTTTACAGC	PCR: EHR16SD/EHR16SR: 345-bp	(Parola and Roux 2000)
Anaplasma					
<i>groESL</i>	Chaperonin protein complex	GroEL-1F GroEL-2R GroEL-2F GroEL-1R-2	CATAGTGATGAAGGAGAGTG CGTTCCTACTAGGAACATCAAC TGTAAGGCGCCTGGTTTCG CTTAAGTCTAGCTCGTC	1 st PCR: GroEL-1F/GroEL-2R: ≈600-bp Nested: GroEL-2F/GroEL-1R-2: 348-bp	(Huang et al. 2005)
Ehrlichia					
<i>dsb</i>	Disulfide oxidoreductase protein	Dsb-330 Dsb-720 Dsb-380	GATGATGTTTGAAGATATSAAACAAAT CTATTTTACTTCTTAAAGTTGATAWAT C ATTTTTAGRGATTTTCCAATACTTGG	1 st PCR: Dsb-330/Dsb-720: 401-bp Hemi-nested: Dsb-330/Dsb-380: 349-bp	(Doyle et al. 2005) (Labruna et al. 2007) (Almeida et al. 2013)
Neorhlichia					
<i>gltA</i>	Citrate Synthase protein	F4b R1b EHR-CS136F EHR-CS778R	CCAGGCTTTATGTCAACTGC CGATGACCAAAACCCA T TTYATGTCYACTGCTGCKTG GCNCCMCCATGMGCTGG	1 st PCR: F4b/ R1b: 800-bp Nested: EHR-CS136F/ EHR-CS778R: 561-bp	(Gofton et al. 2016)
Borrelia					
<i>flaB</i>	Flagellin protein	FLA LL FLA RL FLA LS FLA RS	ACATATTCAGATGCAGACAGAGGT GCAATCATAGCCATTGCAGATTGT AACAGCTGAAGAGCTTGGAATG CTTTGATCACTTATCATTCTAATAGC	1 st PCR: FlaLL/FlaRL: 665-bp Nested: FlaLS/FlaRS: 354-bp	(Stromdahl et al. 2003)
<i>rrs</i>	Bacterial small ribosomal subunit	S5F S13R	GAGGAATAAGCTTTGTAGGA GACGTCATCCTCACCTTCCT	PCR: S5F/ S13R: 746-bp	(Le Fleche et al. 1997)
Hepatozoon					
18S rRNA gene	Eukaryote small ribosomal subunit	HEP1 HEP4 HAM-1 HPF-2	GGTAATTCTAGAGCTAATACATGAGC ACAATAAAGTAAAAACAYTTCAAAG GCCAGTAGTCATATGCTTGTC GACTTCTCCTTCGTCTAAG	PCR: HEP-1F/HEP-4R: 660-680-bp PCR: HAM-1F/HPF-2R: 1750-pb	(Almeida et al. 2013) (Criado-Fornelio et al. 2006)

5.2.4. Phylogenetic analyses

Independent alignments using obtained and GenBank-retrieved sequences were constructed for each gene with the CLUSTAL W algorithm (Thompson et al. 1994), implemented in MEGA 5 (Tamura et al. 2011). Models of evolution were calculated with the same software.

The Anaplasmataceae tree was inferred by means of Minimum Evolution using 1000 bootstrap, and evolutionary distances were computed using the Kimura 2-parameter method with a gamma distribution for 27 sequences. Sequences of a *Wolbachia* endosymbiont (DQ412083), *Neorickettsia* spp. (AF380257 and M73219), and “*Ca. Midichloria mitochondrii*” (AJ566640) rooted the tree.

A Bayesian analysis with 30 sequences of *Hepatozoon* spp. was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) plug-in implemented in Geneious R9 (Kearse et al. 2012), with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 1000th generation; the first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences of *Adelina grylli* (DQ096836), *Haemogregarina balli* (HQ224959), and *Dactylosoma ranarum* (HQ224958) were used as out-group.

Obtained sequences for *Borrelia flaB* gene were aligned with 35 homologous sequences of other *Borrelia* spp. available in GenBank using Clustal X (Thompson et al. 1997), and manually adjusted using GeneDoc (Nicholas et al. 1997). The resulted alignment was used to perform two phylogenetic analyses. A Maximum Parsimony tree was constructed using PAUP version 4.0b10 (Swofford 2002) with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. Subsequently, a Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. *Helicobacter pylori* (KC491788) was aligned as out-group.

GenBank accession numbers for each sequence are embedded in all three phylogenetic trees.

5.3. RESULTS

5.3.1. Ticks

Two hundred and seventy three ticks (three individual and pools of seven, three, five and four larvae; 87 nymphs; 66 females, and 62 males) collected in previous studies were identified as the following species: *Argas* cf. *Argas cucumerinus*, *Argas persicus* (Oken 1818), *Ornithodoros atacamensis* Muñoz-Leal, Venzal & González-Acuña 2016, *Ornithodoros* n. sp. 2 (Muñoz-Leal et al. chapter 1, to be published), *Ornithodoros microlophi* Venzal, Nava & González-Acuña 2013, *Ornithodoros peruvianus* Kohls, Clifford & Jones 1969, *Ornithodoros* n. sp. 1 (Muñoz-Leal et al., chapter 1, to be published), *Ornithodoros* cf. *O. rioplatensis*, and *Ornithodoros spheniscus* Hoogstraal, Wassef, Hays and Keirans 1985 (Table 2). Morphological diagnoses of larval stages of *Ornithodoros* soft ticks collected from reptiles in the current study were identified by the following combination of characters as: 1) *O. atacamensis*: dorsal plate large and pyriform in shape, dorsum provided with 17 pairs of setae, 10 dorsolateral and five central, basis capituli with posterior margin slightly curved (Muñoz-Leal, Venzal, et al. 2016) (Figure 2A); 2) *Ornithodoros lahillei* Venzal, González-Acuña & Nava 2015: dorsal plate subtriangular, with irregular lateral margins and convex posteriorly; dorsum provided with 14 pairs of setae, seven anterolateral, three central and four posterolateral; postcoxal seta absent; hypostome with pointed apex, three rows of denticles in the distal third, then two towards the base, with 16-18 denticles in the first file, 14 to seven in file two, and five to seven denticles in file three (Venzal et al. 2015) (Figure 2B, C, and E); and 3) *O. microlophi*: dorsum provided with 19 – 20 pairs of setae, 13 – 14 dorsolateral, six to eight central, six pairs of sternal setae, and posterior margin of basis capituli straight (Venzal et al. 2013) (Figure 2D).

Upon the material collected from rodents, females of an *Ixodes* sp. morphologically closely related to *Ixodes sigelos* Keirans, Clifford & Corwin, 1976 were identified by the following morphological characters: body outline oval, scapulae pointed; scutum inornate, elongate, with few punctuations, and provided with numerous long setae; ventral surface covered by scattered setae; cornua triangular, porose area oval, auriculae small and triangular; article II of the palp longer than article III, suture conspicuous; coxa I with two triangular spurs, the external larger

than the internal; coxae II-IV with a single short triangular external spur, and spiracular plates rounded (Keirans et al. 1976). A summary of tick species, localities and source of collection is presented in Table 2.

Voucher specimens of slide mounted larvae and *Ixodes* ticks were deposited in the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (CNC) under the following numbers: CNC-3589 (*O. atacamensis*), CNC-3590 (*O. microlophi*), CNC-3592 (*O. lahillei*), and CNC-3598 (*Ixodes* sp.).

Figure 2. Micrographs of *O. atacamensis*, *O. lahillei* and *O. microlophi* larvae. *Ornithodoros atacamensis*: (A) dorsal view (fifth central setae lacking in this specimen); *Ornithodoros lahillei*: (B) dorsal view, (C) dorsal plate, and (E) posteroventral chaetotaxy, note the absence of postcoxal setae; and, *Ornithodoros microlophi*: (D) ventral chaetotaxy. Scale bar is given in millimeters. Abbreviations: Al, anterolateral; C, central; Ca, circumanal; Pc, poscoxal; Pl, posterolateral; Pm, posteromedian.

Table 2. Information on the ticks (L: larvae; N: nymphs; M: males; F: females) analyzed in the current study.

Tick family/species	Number	Slide-mounted larvae	Site of collection/ host	Locality	Date of collection	Source of taxonomic identification
Argasidae						
<i>Argas</i> cf. <i>A. cucumerinus</i>	3L, 15N, 1F	n/a	Rocky cliffs inhabited by seabird	Caleta Vitor	December 2014	Muñoz-Leal et al. (chapter 4)
<i>A. persicus</i>	9N, 13F, 5M	n/a	Henhouse	Calama	December 2014	Muñoz-Leal et al. (chapter 4)
<i>O. atacamensis</i>	22N, 5F, 6M	n/a	Under rocks used by <i>Liolaemus</i> lizards	Pan de Azúcar National Park	November 2014	Muñoz-Leal et al. (chapter 4)
	8L*†	2	<i>Liolaemus bisignatus</i>	Pan de Azúcar National Park	November 2014	This study
	6L*	1	<i>Liolaemus bisignatus</i>	Pan de Azúcar National Park	November 2014	This study
<i>O. peruvianus</i>	8F, 4M	n/a	Cave inhabited by <i>Desmodus rotundus</i>	Pan de Azúcar National Park	December 2015	Muñoz-Leal et al. (chapter 4)
	24N, 8F, 17M	n/a	Cave inhabited by <i>Desmodus rotundus</i>	Taltal	December 2014	Muñoz-Leal et al. (chapter 4)
<i>O. spheniscus</i>	10F, 10M	n/a	Near <i>Spheniscus humboldtii</i> nests	Pan de Azúcar National Park	December 2015	Muñoz-Leal et al. (chapter 4)
	11F, 11M	n/a	Near <i>Pelecanus thagus</i> nests	The Dead Islet, Mocha Island	December 2015	Muñoz-Leal et al. (chapter 4)
<i>O. microlophi</i>	7N, 1M, 1H	n/a	Inside <i>Microlophus</i> lizards burrows	Pampa del Tamarugal National Reserve	November 2014	Muñoz-Leal et al. (chapter 4)
	5L*†	1	<i>Microlophus theresioides</i>	Pampa del Tamarugal National Reserve	December 2015	This study
	2L*	1	<i>Microlophus theresioides</i>	Pampa del Tamarugal National Reserve	December 2015	This study
	3L*	n/a	<i>Microlophus theresioides</i>	Pampa del Tamarugal National Reserve	December 2015	This study
	1L	n/a	<i>Phyllodactylus gerrhopygus</i>	Pampa del Tamarugal National Reserve	December 2015	This study
<i>Ornithodoros</i> cf. <i>O. rioplatensis</i>	2H, 5M, 3N	n/a	Under rocks sheltering <i>Microlophus</i> lizards	Santa María Island	November 2014	Muñoz-Leal et al. (chapter 4)
	1N, 2M, 7F	n/a	Under volcanic rocks	Nevados de Chillán	November 2016	Muñoz-Leal et al. (chapter 4)

* pool/ † one tick separated to perform mitochondrial 16S rRNA PCR; n/a: not available.

Table 2. (Continued).

Tick family/species	Number	Slide-mounted larvae	Site of collection/ host	Locality	Date of collection	Source of taxonomic identification
Argasidae						
<i>O. lahillei</i>	8L*	3	<i>Callopistes maculatus</i>	Las Chinchillas National Reserve	November 2014	This study
	10L*†	2	<i>Callopistes maculatus</i>	Las Chinchillas National Reserve	November 2014	This study
<i>Ornithodoros</i> n. sp. 1	7L*	n/a	<i>Octodon degus</i>	Las Chinchillas National Reserve	November 2014	Muñoz-Leal et al. (chapter 3)
	3L*	n/a	<i>Octodon degus</i>	Las Chinchillas National Reserve	November 2014	Muñoz-Leal et al. (chapter 3)
	5L*	n/a	<i>Octodon degus</i>	Las Chinchillas National Reserve	November 2014	Muñoz-Leal et al. (chapter 3)
	4L*	n/a	<i>Octodon degus</i>	Las Chinchillas National Reserve	November 2014	Muñoz-Leal et al. (chapter 3)
	8N, 2F, 6M	n/a	<i>Octodon degus</i> burrows	Las Chinchillas National Reserve	November 2014	Muñoz-Leal et al. (chapter 3)
<i>Ornithodoros</i> n. sp. 2	1N, 2F, 1M	n/a	Bird/rodent burrow	Bosque de Fray Jorge National Park	November 2014	Muñoz-Leal et al. (chapter 3)
Ixodidae						
<i>Ixodes</i> cf. <i>I. auritulus</i>	1L†, 4N†	n/a	<i>Troglodytes musculus</i>	Bahia Eugenia, Navarino Island	January 2017	This study
<i>Ixodes</i> cf. <i>I. sigelos</i>	2N*, 1F†	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	7L*, 2N*	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	9L*	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	7L*, 1F†	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	6L*, 3N*, 1F	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	10L*, 2N*	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study

* pool/ † one tick separated to perform mitochondrial 16S rRNA PCR; n/a: not available.

Table 2. (Continued).

Tick family/species	Number	Slide-mounted larvae	Site of collection/ host	Locality	Date of collection	Source of taxonomic identification
Ixodidae						
<i>Ixodes</i> cf. <i>I. sigelos</i>	3N*	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	1F	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	2N*	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	3N*, 1F	n/a	<i>Phyllotis darwini</i>	Bosque de Fray Jorge National Park	November 2014	This study
	1F	n/a	<i>Oligoryzomys longicaudatus</i>	Bosque de Fray Jorge National Park	November 2014	This study
	2N*, 1F	n/a	<i>Octodon degus</i>	Bosque de Fray Jorge National Park	November 2014	This study
	3N*	n/a	<i>Octodon degus</i>	Bosque de Fray Jorge National Park	November 2014	This study
	3N*	n/a	<i>Octodon degus</i>	Bosque de Fray Jorge National Park	November 2014	This study
	3N*	n/a	<i>Octodon degus</i>	Bosque de Fray Jorge National Park	November 2014	This study
	43N†, 7F†	n/a	<i>Abrothrix longipilis</i>	Mocha Island	December 2015	This study

* pool/ † one tick separated to perform mitochondrial 16S rRNA PCR; n/a: not available.

5.3.2. Molecular tools

5.3.2.1. Ticks

While the three *Ornithodoros* species submitted to PCR targeting the mitochondrial 16S rRNA gene yielded a 426-bp fragment (excluding primer sequences), variable amplicon sizes were obtained for *Ixodes* spp., as follow: 411-bp, *Ixodes* sp (BE); 404-bp, *Ixodes* sp. (BFJNP); 409-bp, *Ixodes* sp. (MI); 403-bp, *I. taglei* (Santa Juana); 405-bp, *I. taglei* (Los Angeles); and, 409-bp, *I. uriae*. Unfortunately, fourteen *I. neuquenensis*, two *I. taglei*, and four *I. uriae* resulted negative to the initial PCR targeting the mitochondrial 16S rRNA gene, and no sequence was obtained for the sole PCR-positive nymph of *I. neuquenensis*. Consequently these ticks were discarded from subsequent analyses.

Molecular tools confirmed morphological diagnoses for soft ticks, since most sequences of mitochondrial 16S rDNA matched 100% (426-bp), 96.9% (355/366-bp), and 99.3% (423/426-bp) with conspecific sequences of *O. atacamensis* (KT894587), *O. lahillei* (KP403288) an *O. microlophi* (JX455899). In turn, one female of *I. taglei* from Santa Juana, and two from Los Angeles, yielded sequences 98.9% (354/358-bp), and 99.2% (357/360-bp), respectively, identical to *I. taglei* from Chile (KU729880). Moreover, one male, one female, and one nymph of *I. uriae* yielded a consensus 99.5% (407/409-bp) identical with one of the conspecific sequences deposited in GenBank (D88304). Controversial results were observed for sequences from *Ixodes* ticks collected in BFJNP and MI. One haplotype was confirmed as consensus per each locality, with 95.6% (391/409-bp) of nucleotide identity between them. Moreover, although both sequences were 95.8% (390/407-bp), and 97.1% (398/410-bp) identical with *I. sigelos* from Argentina (HM014413), they also matched with more than 95% of identity with *Ixodes abrocomae* Lahille 1916 (GU188043), and GU188044) and *I. stilesi* (DQ061292). On the other hand, the mitochondrial 16S rDNA consensus sequence of *Ixodes* ticks collected in Bahia Eugenia was 99.0% (407/412-bp) identical with *Ixodes auritulus* Neumann 1904 (AF549845). Because morphological and molecular identification of *Ixodes* ticks

collected in BFJNP and MI was only partially related, it was opted to treat the examined specimens as *Ixodes* cf. *I. sigelos*. Similarly, immature stages of *Ixodes* collected on a bird in BE were only identified by molecular tools, so they were treated as *Ixodes* cf. *I. auritulus*.

5.3.2.2. Anaplasmataceae, *Borrelia* and *Hepatozoon*

Overall, DNA of Anaplasmataceae, *Borrelia* and *Hepatozoon* was amplified only in four of the 15 analyzed tick species. PCR screenings were successful in *Ornithodoros atacamensis*, *O. spheniscus*, *Ixodes* cf. *I. sigelos* and *Ixodes* cf. *I. auritulus*. PCRs targeting the Anaplasmataceae 16S rRNA gene yielded sequences of 306-bp with identities 98-100% identical with organisms of genus *Ehrlichia*, *Anaplasma*, and *Neoehrlichia*. Unfortunately, no sequences of *Anaplasma*-positive *O. spheniscus* from Pan de Azúcar Island were obtained. DNA of *Borrelia* was detected in all four tick species; however not a clean sequence was obtained for *O. atacamensis*. Remarkably, *flaB* haplotypes retrieved from *Ixodes* spp. collected in BFJNP and MI were identical. In turn, only *O. spheniscus* was negative for *Hepatozoon* screenings, and although *Ixodes* cf. *I. sigelos* from Mocha Island yielded expected size amplicons, sequencing attempts were unsuccessful (Table 3). A second PCR was intended to molecularly characterize fragments of *groESL*, *dsb* and *gltA* genes for *Anaplasma*-, *Ehrlichia*- and “*Ca. Neoehrlichia*”-positive ticks respectively. No amplification was observed in tick-extracted DNA samples. No amplicons were obtained for *Ca. Neoehrlichia gltA* and *Borrelia rrs* PCRs.

Remarkably, Anaplasmataceae DNA was detected in all stages of *O. spheniscus*, in 20% of *O. atacamensis* specimens, and in 17.4% of *Ixodes* ticks collected in BFJNP. In turn, *Borrelia* DNA was amplified in 2 - 20% percent for each positive species, and prevalence of *Hepatozoon* did not exceed 9% (Table 3).

Table 3. Collection localities of soft and hard ticks positive for Anaplasmataceae, *Borrelia* and *Hepatozoon* spp.. The identity of the first 100% BLAST cover query match is indicated.

Positive species Locality	Feeding status	Host/source of collection	Detected microorganism (gene)		
			Anaplasmataceae (<i>rrs</i>)	<i>Borrelia</i> (<i>flaB</i>)	<i>Hepatozoon</i> (18S rRNA gene)
ARGASIDAE					
<i>Ornithodoros atacamensis</i> Pan de Azúcar National Park	Unengorged	Under rocks frequented by <i>Liolaemus</i> lizards	98.7% (302/306-bp) <i>Ehrlichia</i> sp. strain WHBMXZ-40 (KX987325) P: 20% (4N, 1F, 2M)	No sequence obtained P: 2.7% (1M)	98.0% (1638/1671-bp) <i>Hepatozoon</i> sp. DG1 (FJ719813) P: 2.7% (1M)
<i>Ornithodoros spheniscus</i> Pan de Azúcar Island	Unengorged	Under rocks near <i>Spheniscus humboldtii</i> nests	Not sequence obtained P: 100% (10M, 10F)	98.3% (623/634-bp) <i>Borrelia turicatae</i> (CP015629) P: 5% (1F)	_____
The Dead Islet, Mocha Island	Unengorged	Under rocks near <i>Pelecanus thagus</i> nests	98.7% (302/306-bp) <i>Anaplasma</i> sp. isolate HN709 (KX505299) P: 100% (11M, 11F)	_____ —	_____
IXODIDAE					
<i>Ixodes cf. I. auritulus</i> Bahía Eugenia, Navarino Island	Engorged	<i>Troglodytes musculus</i>	_____ _____	93.8% (288/307-bp) <i>Borrelia garinii</i> (KR782221) P: 20% (1N)	_____
<i>Ixodes cf. I. sigelos</i> Bosque de Fray Jorge National Park	Engorged	<i>Phyllotis darwini</i>	99.0% (303/306-bp) <i>Candidatus</i> Neoehrlichia lotoris (EF633744) P: 17.4% (3F, 3N*)	98.0% (301/307-bp) <i>Borrelia</i> sp. clone ISIG1 (KX417768) P: 8.7% (9L*, 3N*)	98.3% (1503/1529-bp) <i>Hepatozoon</i> sp. AO5 (FJ719818) P: 8.7% (7L*, 1F)
	Slightly engorged	<i>Phyllotis darwini</i>	_____ _____	_____ —	98.2% (1503/1531-bp) <i>Hepatozoon</i> sp. AO5 (FJ719818) P: 8.7% (10L*, 3N*)
Mocha Island	Slightly engorged	<i>Abrothrix longipilis</i>	_____ _____	98.0% (301/307-bp) <i>Borrelia</i> sp. clone ISIG1 (KX417768) P: 16% (8N)	Not sequence obtained P: 6% (3N)

* pool of ticks/ L: larva; N: nymph; F: female; M: male; P: prevalence

5.3.3. Phylogenetic analyses

5.3.3.1. Anaplasmataceae

Although not every branch was well supported, the phylogenetic analysis for Anaplasmataceae family clearly grouped monophyletic clades of *Anaplasma*, *Ehrlichia*, and “*Ca. Neoehrlichia*” spp. Remarkably, and despite the shortness of the obtained sequences, the *Anaplasma* sp. detected in *O. spheniscus*, the *Ehrlichia* sp. from *O. atacamensis*, and the “*Ca. Neoehrlichia*” sp. characterized from *Ixodes* cf. *I. sigelos* collected in BFJNP, clustered within each corresponding clade (Figure 3).

5.3.3.2. *Borrelia*

By the phylogenetic analysis of a short fragment (≈300-pb) of the flagellin-encoding gene, genospecies belonging to both the LDG and RFG borreliae were clearly separated in two major clades (Figure 4). The *flaB* haplotype retrieved from *O. spheniscus* clusters basally within a clade composed by a *Borrelia* sp. characterized from *Ornithodoros kelleyi* Cooley and Kohls, 1941, *Borrelia hermsi* and *Borrelia turicatae*. On the other hand, sequences from *Borrelia* spp. obtained from *Ixodes* spp. grouped with other LDG borreliae. Identical haplotypes retrieved from *Ixodes* cf. *I. sigelos* ticks from BFJNP and MI clustered as a sister group to a *Borrelia* sp. characterized from *I. sigelos* collected in Argentina. Remarkably, after BLAST pairwise comparisons a *flaB*, an unpublished sequence from *Borrelia garinii* obtained from *Ixodes ricinus* (Linnaeus, 1758) collected in Poland was the most identical sequence, yet with more than 6% of genetic divergence. Consequently, in the current phylogenetic analysis the *flaB* haplotype obtained from *Ixodes* cf. *I. auritulus* forms an isolated branch basally positioned with respect to other LDG borreliae.

5.3.3.3. *Hepatozoon*

Overall, higher support values were obtained for the topology of the *Hepatozoon* spp. phylogenetic tree. While both haplotypes retrieved from *Ixodes* cf. *I. sigelos* collected on *P. darwini* in BFJNP grouped as a sister clade of an *Hepatozoon* group (*Hepatozoon* sp. AO5, AO6, AO12, AS7, and AS15) characterized from

tissues of other Chilean rodents (*Abrothrix* spp.) (Merino et al. 2009), the *Hepatozoon* sequence obtained from *O. atacamensis* appeared as an independent clade, closely related to a conspecific agent characterized from *D. gliroides* (*Hepatozoon* sp. DG1, and DG2) (Figure 5).

Figure 3. Minimum Evolution tree inferred for a partial fragment of the Anaplasmataceae family 16S rRNA gene. The positions of the microorganisms characterized from ticks of the current study are highlighted in bold orange, blue and green. Abbreviations: *Ca.*, *Candidatus*; BFJNP, Bosque de Fray Jorge National Park.

Figure 4. Phylogenetic tree inferred for a partial fragment of the *Borrelia flaB* gene. The positions of the microorganisms characterized from ticks of the current study are highlighted in bold. Maximum Parsimony bootstrap and Bayesian posterior probability values are indicated in front of each clade, respectively.

Figure 5. Bayesian phylogenetic tree inferred for the sequences of *Hepatozoon* 18S rRNA gene. The positions of the sequences obtained in the current study are highlighted in red bold.

5.4. DISCUSSION

5.4.1. Ticks

By a morphological and a molecular approach all the soft tick larvae collected in this study were conclusively identified as *O. atacamensis*, *O. lahillei*, or *O. microlophi*. However, molecular results were not reflected in observed morphological traits for adults of *Ixodes* collected in BFJNP and MI, since genetic identities were almost 5.0% divergent between each other and also with other congeneric and sympatric species. This incongruence might be explained by the little knowledge on intraspecific genetic variability for populations of Chilean *Ixodes*. However, it might also indicate the occurrence of an underestimated diversity. Genetic identity of the sole larval and nymphal instars of the *Ixodes* sp. collected in BE was almost equal to *I. auritulus*. Because the identification of this material lacked a morphological approach, and because *I. auritulus* likely compose a group of morphologically closely related species (Arthur 1958), we kept the diagnosis as *Ixodes* cf. *I. auritulus*. Noteworthy, the site where immature stages of *Ixodes* cf. *I. auritulus* were collected is relatively close to (≈ 300 km) the type locality of the species (Punta Arenas) (Neumann 1904), so the partial sequence of the mitochondrial 16S rRNA gene obtained from herein collected ticks could potentially correspond to *I. auritulus* sensu stricto.

As *Ixodes* spp. ticks were collected on their hosts and showed a degree of engorgement, positivity of Anaplasmatataceae, *Borrelia* or *Hepatozoon* does not imply that these ticks are indeed vectors or reservoirs of the detected agents. However, it constitutes direct evidence pointing their potential role for harboring or even transmitting the detected microorganisms.

5.4.2. Anaplasmatataceae

Recent research on Anaplasmatataceae bacteria associated to ticks has shown the occurrence of new haplotypes (Carpi et al. 2011, Gofton et al. 2015), and candidates for new species (Eshoo et al. 2015, Gofton et al. 2016), which reflects an underestimated diversity of bacteria of this family among the Ixodoidea. Three 16S rDNA sequences of Anaplasmatataceae microorganisms phylogenetically related with

the genera *Anaplasma*, *Ehrlichia* and “*Ca. Neoerhlichia*”, were obtained from *O. spheniscus*, *O. atacamensis*, and *Ixodes* cf. *I. sigelos*, respectively. In the phylogenetic analysis, corresponding sequences clustered within a defined genus; however genetic characterization using genus-specific primers to amplify partial fragments of the genes *groELS*, *dsb* and *gltA* (Doyle et al. 2005, Huang et al. 2005, Labruna et al. 2007, Almeida et al. 2013, Gofton et al. 2016) were unsuccessful. To match a bacterial genus by the comparison of a short 16S rDNA sequence, but to show negative results in genus-specific PCR attempts, might be reflecting a degree of genetic divergence between the herein characterized microorganisms with congeneric agents of already known phylogenetic positions.

Noteworthy, all *O. spheniscus* specimens analyzed in the current study were prevalent for a microorganism phylogenetically related to *Anaplasma* representatives. High prevalence of infection has been evidenced for other Anaplasmataceae representatives, such as bacteria of genus *Wolbachia*, that merge their biological cycles in endosymbiotic associations with arthropods (Riegler and O'Neill 2006). In this way, the *Anaplasma*-like agent detected in *O. spheniscus* could represent a previously undocumented endosymbiont.

Even though possible, all the above stated must be carefully addressed, since the herein analyzed 16S rRNA Anaplasmataceae amplicon is rather short, and might result in inconsistent signal within a phylogenetic reconstruction with closely related organisms. Larger or full-length sequences for the *rrs* codifying regions of the microorganisms detected in *O. atacamensis*, *O. spheniscus* and *Ixodes* spp. are needed in order to accurately define their phylogenetic position within the Anaplasmataceae.

5.4.3. *Borrelia*

Phylogenetic analyses based exclusively in *flaB* sequences have demonstrated to be useful in order to separate different *Borrelia* species (Fukunaga et al. 1996). Even though in this study only a short fragment of this gene was obtained for each characterized *Borrelia*, the insertion of these sequences into a phylogenetic context retrieved a logic topology for the genus, clearly separating LDG from RFG borreliae.

Our results are in the line with other *Borrelia* detections published for the South American fauna of ticks (Parola et al. 2011, Barbieri et al. 2013, Nava et al. 2014, Sebastian et al. 2016, Dall'Agnol et al. 2017), and demonstrates that the diversity of spirochetes of this genus is currently underestimated for the region. However, discounting the findings of Parola et al. (2011), efforts to detect *Borrelia* species in South American ticks have been focused in *Ixodes* representatives, which constitute main reservoirs of LDG borreliae (Kurtenbach et al. 2006). In an unprecedented attempt, the current study included ten South American soft tick species for *Borrelia* DNA screening. Although *O. atacamensis* also yielded expected size amplicons, only *O. spheniscus* products resulted in a reliable sequence. *Ornithodoros spheniscus* is a seabird-associated parasite and, remarkably, most identical sequence for the herein obtained *flaB* haplotype did not correspond to any RFB characterized from a bird-associated soft tick (i. e. *B. anserina*, *Borrelia* sp. from *Ornithodoros capensis* Neumann 1901), and rather matched *B. turicatae* (98.3%, 623/634-bp), a species harbored by the mammal-associated tick *Ornithodoros turicata* (Dugès 1876) (Davis 1943). The current phylogenetic analysis based on *flaB* sequences confirmed this intriguing result, and while further genetic characterizations are needed for a conclusive identification, it suggests that bird-associated RFB are composed by different independent lineages.

On the other hand, two identical *flaB* haplotypes of LDG borreliae detected in *Ixodes* cf. *I. sigelos* collected in two distanced Chilean localities were phylogenetically closely related to *Borrelia* sp. haplotype Patagonia, which was recently detected in *I. sigelos* and *Ixodes* cf. *I. neuquenensis* from Southern Argentina (Sebastian et al. 2016). As observed by Sebastian et al. (2016), this putative new *Borrelia* genospecies conforms a monophyletic group that also includes *B. chilensis*, a species associated to *I. stilesi* in Chile. For instance, natural reservoirs of this monophyletic group of borreliae seems to be composed by South American endemic cricetid rodents (Rodentia: Cricetidae), and their associated *Ixodes* ticks, since all positive ticks have been collected upon representatives of genera *Abrothrix* (this study), *Phyllotis* (Sebastian et al. 2016, this study), and *Oligoryzomys* (Ivanova et al. 2013). Such a delimited group of borreliae genospecies was not observed when genetically comparing and subsequently inserting the *Borrelia* haplotype retrieved from *Ixodes* cf. *I. auritulus* in the phylogenetic analysis, since an isolated and basally positioned branch over the all the other species was observed, suggesting the

occurrence of a putative new genospecies (Figure 4). *Ixodes auritulus* is likely to compose a widely distributed group of species (Arthur 1958), and although *Borrelia*-infected populations have been documented to occur in Northern latitudes of the American Continent (Morshed et al. 2005, Scott et al. 2010), the diversity of *Borrelia* spirochetes associated to this alleged species complex is still poorly understood. A set of *rrf-rrl* intergenic spacers sequences constitute the only available genetic data on *I. auritulus*-characterized borreliae from Canada, which to date defines in a broad sense the occurrence of *B. burgdorferi* s. l. in this tick species (Morshed et al. 2005, Scott et al. 2010). Unfortunately, apart from the herein obtained results, sequences of the *Borrelia flaB* gene characterized from *I. auritulus* ticks are inexistent, a fact that precluded any genetic comparison.

5.4.4. *Hepatozoon*

The occurrence of *Hepatozoon* in wild vertebrates and ticks from South America has been a focus of study in recent years. While a conserved gene (i. e. eukaryote 18S ribosomal gene) has been used to detect, and further assess the phylogenetic position of the detected agents, novelties have not been absent: several new haplotypes and putative new species have been documented even by the analyses of partial sequences (Forlano et al. 2005, Criado-Fornelio et al. 2006, Wolf et al. 2016, Soares et al. 2017). Here, sequences >1500-bp long were retrieved from engorged *Ixodes* from BFJNP, and from one unengorged *O. atacamensis* in PANP. Phylogenetic analyses pointed a defined position in individual branches of the tree (Figure 5), which could be indicative that these new haplotypes indeed represent new species. Interestingly, the *Hepatozoon* spp. characterized from *Ixodes* spp. cluster as a sister group with near relatives found in tissues of cricetid small-mammals (Rodentia: Cricetidae) of genus *Abrothrix* from Chile (Merino et al. 2009). Hosts of the herein positive *Ixodes* ticks belong to the genus *Phyllotis*, classified within the Cricetidae as well (Iriarte 2008), a taxonomical relationship which in addition to the topology of the obtained phylogenetic tree, suggests the occurrence of a natural group of *Hepatozoon* spp. associated to this family of rodents and their ticks. However, with current evidence, the possible role of *Ixodes* ticks as definitive hosts for these putative *Hepatozoon* spp. must still be assessed.

On the other hand, care should be taken when inferring possible hosts for the *Hepatozoon* haplotype retrieved from an unengorged male of *O. atacamensis*. Ticks of this family (Argasidae) are able to starve for a long period of time (Sonenshine and Roe 2014), so no reliable conclusions on the provenance of the detected *Hepatozoon* DNA can be taken. Moreover, postlarval stages of *Ornithodoros* ticks are somewhat generalist feeders (Oliver 1989), and exploit different classes of vertebrates. While *O. atacamensis* was described in association with reptiles, other hosts must not be discarded, since several small-mammals inhabits the same ecosystems where ticks of this species can be found (Iriarte 2008). Apart from this unanswered question, this is the first time that DNA of *Hepatozoon* is detected in a soft tick species. Previous results published by Bennett et al. (1992) were the only evidence that ticks of the Argasidae family could play a role in the life cycle of *Hepatozoon* spp., since oocysts of *Hepatozoon atticorae* were observed in the haemolymph cells of *Ornithodoros peringueyi* Bedford & Hewitt, 1925, a soft tick associated to Cliff Swallows in South Africa. For confirming that *O. atacamensis* is a definitive host of the detected *Hepatozoon* sp., experimental work in order to visualize oocyst forms in tick cells must be performed.

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6. THE *Ornithodoros capensis* GROUP (ACARI: ARGASIDAE) OF THE NEOTROPICAL REGION: GENETIC IDENTITY, MORPHOLOGY, AND GEOGRAPHICAL DISTRIBUTION OF FOUR SPECIES IN SOUTH AMERICA

6.1. INTRODUCTION

The *Ornithodoros capensis* group (Acari: Argasidae) rose upon morphological affinities observed in adults and larval stages of bird-associated soft ticks. The type species of the group, *Ornithodoros capensis*, was described by Neumann in 1901 upon the examination of specimens collected by C. F. Lounsbury on guano from a colony of South African penguins (Neumann 1901). By the 1900s, the diversity of Argasidae was resumed to eleven species, namely - in chronologic order of description - *Ornithodoros savingyi* (Adouin, 1826), *Ornithodoros coriaceus* Koch 1844, *Ornithodoros erraticus* (Lucas 1849), *Ornithodoros talaje* (Guérin-Ménéville 1849), *Ornithodoros turicata* (Dugès 1876), *Ornithodoros moubata* (Murray, 1877), *Ornithodoros rudis* Karsch 1880, *Otobius megnini* (Dugès 1883), *Ornithodoros coniceps* Canestrini 1890, *Ornithodoros canestrinii* (Birula 1895), and *Ornithodoros papillipes* (Birula 1985). Considering the morphology of adult stages of all these species, Neumann attributed the name *O. talaje* var. *capensis* to the South African ticks, however he explicitly recognized that they were more closely related to *O. coniceps* (named by him as *O. talaje* var. *coniceps*) (Neumann 1901). Few years latter Nuttall et al. (1908) examined and compared original allotments made by Lounsbury, and conclude that the same species was present in St Paul's Rocks (São Pedro e São Paulo Archipelago, Brazil).

Until 1997, descriptions of *Ornithodoros* morphologically *affinis* to *O. capensis* gather eleven species. Almost twenty years after Neumann's description, Chamberlin

(1920) described *Ornithodoros amblus* Chamberlin 1920, collected from seabird guano at Chinchas Islands, Peru. Later, in series of papers summarizing the systematics of the Ornithodorinae, Kohls et al. (1965) presented designs of the *O. capensis* larva, reared from females collected in South African Penguin colonies, and described a closely related species, *Ornithodoros denmarki* Kohls, Sonenshine & Clifford 1965, based on larval stages reared from females collected in the Florida State (the United States) (Denmark and Clifford 1962). Remarkably, Kohls et al. (1965) stated that adults of *O. denmarki* are extremely similar to *O. capensis*, and that it was impossible to find clear morphological characters to separate both species without examining larval stages. In France, Vermeil and Marguet (1967) described the larva of *Ornithodoros maritimus* Vermeil & Marguet 1967, which validity as species was confirmed years latter by Hoogstraal et al. (1976). Two years after, Hoogstraal (1969) described the adults of *Ornithodoros muesebecki* Hoogstraal 1969 from Boobie's nesting ground in the Arabian gulf, and further gave morphological details of its immature stages (Hoogstraal et al. 1970). In 1973 Kitaoka and Susuki described *Ornithodoros sawaii* from Japan (Kitaoka and Suzuki 1973). Finally, *Ornithodoros collocalie* Hoogstraal, Kadarsan, Kaiser & Van Peen 1974 was described from swift swallows at Java Island (Hoogstraal et al. 1974); *Ornithodoros cheikhi* Vermeil, Marjolet & Vermeil 1997 (Vermeil et al. 1997) from nesting terns in Mauritania, and *Ornithodoros yunkerii* Keirans, Clifford & Hoogstraal 1984 and *Ornithodoros spheniscus* Hoogstraal, Wassef, Hays & Keirans 1985, were subsequently described associated to seabirds in South American shores of the Pacific Ocean (Keirans et al. 1984, Hoogstraal et al. 1985).

Complex of species are arrays of phenotypically closely related taxa that may conform or not a monophyletic group. Molecular data has been advantageous in order to evaluate systematic relationships between species mainly because of the extent of the data set that is submitted to analysis (Hillis 1987). Applied to the study of ticks, molecular tools have played a fundamental role unveiling species previously thought to represent a defined taxon, as assemblages of entities sharing a common ancestor (Beati et al. 2013, Hekimoğlu et al. 2016). On the other hand, paraphyly of a complex has also been proven (Xu et al. 2003). Particularly, phylogenetic studies based on mitochondrial 16S rDNA sequences have put in evidence that the *O. capensis* group might be composed by several undescribed species (Dupraz et al. 2016), and that the variability of haplotypes in a specific location might correspond to

the occurrence of several species converging as a result of seabird-spreading (Ushijima et al. 2003, Gómez-Díaz et al. 2012). Although plausible, considering that *O. capensis* sensu lato (s. l.) is a morphologically closely related group of species (Hoogstraal 1985), any hypothesis concerning to the diversity of ticks of this group based on genetic data, might be carefully addressed. While genetic identities of soft ticks associated to seabirds are abundant in online databases, few of them have been accompanied by a proper morphological identification. Because representatives of *O. capensis* s. l. might occur in sympatry, and because slight morphological differences often separate species within this group, specific diagnoses relying on historically documented distributions and exclusive examination of adult stages, might lead to misidentifications.

The main purpose of this study was to assign a genetic identity to seabird *Ornithodoros* collected over an extended coastal line across the shores of Chile and Peru, and some insular territories of Brazil, by obtaining sequences of tick mitochondrial genes. Furthermore, to reinforce their genetic identities, several genes of associated *Coxiella*-like endosymbiont were also obtained. As for all species, ticks of this group have a single evolutionary history, therefore congruence between two systematic approaches might correspond to strong evidence that the underlying phylogenetic scenario has been uncovered (Hillis 1987). Thus, genetic data on ticks and *Coxiella*-like endosymbionts was contrasted by independent phylogenetic analysis. As these mutualistic microorganisms have demonstrated to be specific in ticks (Duron et al. 2015, 2017), it would be expected that phylogenetic reconstructions of both groups will follow a similar pattern of divergence, resulting in congruent evolutionary histories.

6.2. MATERIAL AND METHODS

6.2.1. Sites of study and collection of ticks

The material analyzed in this study was collected between November 2014 to August 2017, in 18 localities distributed in the seaboard and insular territories of three South American countries as follows: 1) Brazil: Queimada Grande Island (São Paulo state, 24°29'S; 46°40'W), and São Pedro e São Paulo Archipelago (Pernambuco state, 0°55'N; 29°20'W); 2) Chile: The Dead Islet (Mocha Island, Biobio region, 38°25'S; 73°55'W); Pájaros Island (Coquimbo region, 29°35'S; 71°32'W); Chañaral Island (Atacama region, 29°02'S; 71°34'W); Pan de Azúcar National Park (Atacama region, 26°17'S; 70°38'W); Taltal (Antofagasta region, 25°23'S; 70°30'W); Santa María Island (Antofagasta region, 23°26'S; 70°36'W); Tocopilla (Antofagasta region, 22°05'S; 70°11'W), and Caleta Vitor (Arica and Parinacota region, 18°45'S; 70°20'W); and 3) Peru: Punta Picata (Tacna department, 17°51'S; 71°05'W); Piedra Blanca (Arequipa department, 16°09'S; 73°49'W); Chala (Arequipa department, 15°52'S; 74°14'W); Tanaka (Arequipa department, 15°44'S; 74°27'W); San Juan de Marcona (Ica department, 15°21'S; 75°10'W); Paracas (Ica department, 13°50'S; 76°15'W); Trujillo (Trujillo department, 8°07'S; 79°03'W), and Foca Island (Piura department, 5°12'S; 81°12'W) (Figure 1).

The search for ticks was actively conducted during daytime lifting rocks and in crevices near seabird resting places. While the material collected in Caleta Vitor was preserved in 90 - 95% ethanol, all other ticks were brought alive to laboratory. Upon arrival, ticks were separated by stage and sex. As in general the objectives of this study were to genetically identify and morphologically compare different species of seabird collected in all the above stated localities, only several specimens per identified morphotype were selected to perform the analyses.

Figure 1. Localities where seabird soft ticks were collected in South America.

6.2.2. Morphological study

6.2.2.1. Identification of adult stages

In soft tick taxonomy, morphology of the larval stage has shown to be informative in order to separate species (Klompen 1992). However, with the exception of *O. capensis* s. s., morphological traits of the larval stage of *O. capensis* s. l. species currently described for the Neotropical Zoogeographic Region are highly similar (Kohls et al. 1965, Clifford et al. 1980, Keirans et al. 1984, Hoogstraal et al. 1985). Thus, following original descriptions for neotropical species (Clifford et al. 1980, Keirans et al. 1984, Hoogstraal et al. 1985), and scanning electron microscopy micrographs available online (<http://www.discoverlife.org>), it was opted to follow an inverted taxonomical approach, and rather use the morphology of adult forms to achieve specific diagnoses. To clearly define anatomical structures showing interspecific variation, we followed the terminology for dorsal disks proposed by Sonenshine (1962), with modifications. South American species of *O. capensis* s. l. belong to the *Alectorobius* Pocock 1907 subgenus (Clifford et al. 1980, Keirans et al. 1984, Hoogstraal et al. 1985), so nomenclature of dorsal disks was adapted following the anatomy of adult representatives of the *O. talaje* group (Figure 2) (since *O. talaje* sensu stricto [s. s.] is the type species of the *Alectorobius* subgenus). As it was impossible to examine the type series of *O. talaje* s. s., the consensus for the disposition and number of dorsal disks was achieved by the examination of the following adults specimens of the group deposited at the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva”, University of São Paulo, São Paulo Brazil: *Ornithodoros atacamensis* Muñoz-Leal, González-Acuña & Venzal 2016 from Chile (CNC-3262), *Ornithodoros guaporensis* Nava, Venzal & Labruna 2013 from Bolivia (CNC-2305); *Ornithodoros rioplatensis* Venzal, Estrada-Peña & Mangold 2008 from Uruguay (CNC-3254), and *Ornithodoros puertoricensis* Fox 1947 from Panama (CNC-3255). Additional observations of species from this group were made from the micrographs given by Cooley and Kohls (1944) and illustrations from Brumpt (1922).

When possible, up to ten females, males or both sexes were selected upon different identified forms in order to examine the topology and macro anatomy of

dorsal disks, mammillae and body outline. A draw of these observations for each identified species was based in micrographs taken with a SteREO Discovery V12 stereomicroscope, and the software ZEN 2 pro.

After identifying all the material, two females per species per locality were fed in naïve chickens, individually accommodated within glass tubes with autoclaved sand, and placed inside an incubator at 25°C and 80 – 85% of relative humidity, in order to obtain unfed larval offspring. In the cases when both females succeeded to oviposit, only one of both cohorts of larvae was selected for a morphological and morphometrical analysis. Egg-laying females that survived after the oviposition, or failing that, pools of larvae from females that died after oviposition were submitted to DNA extraction. Males or nymphs were submitted to DNA extraction only for localities where no females were collected in the field, or when egg-laying females perished before oviposition.

Figure 2. Pattern of dorsal disks of a hypothetical *Alectorobius* species. Note that only the Anteromedian, Median disk and the Posteromedian file are unpaired. Anterolateral line is composed by disks I, II, III and IV, and each intermedian pair is composed by two disks, separated or joining between each other.

6.2.2.2. Larval stage

One week after the eclosion, 17 cohorts of ten and one of six unfed larvae, each obtained from a unique female, were separated and killed in hot water to perform a morphological analysis. Larvae were clarified in a 20% KOH solution, during 20 – 30 min, mounted in slides using Hoyer's medium, and dried in an incubator at 25°C during three days. Subsequently, morphological characters were observed through light microscopy (Olympus BX40 optical microscope), photographed, and measured above micrographs using the software Image-Plus Pro v5.1. Measurement are given in micrometers followed by the standard deviation with minimal and maximum values between parentheses.

6.2.2.3. Principal Component Analysis

In order to address the existence of morphological variability between the identified species, obtained measures for sets of unfed larvae per species were independently submitted to a principal component analysis (PCA) based on Pearson correlation matrix using 59 characters for *O. amblus*, *O. spheniscus*, *Ornithodoros* cf. *O. yunker*, and 69 for *O. capensis*. Measurements of ten *O. capensis* larvae from Queimada Grande Island published in a previous study (Muñoz-Leal et al. 2017) were also included for the PCA of this species. A fifth comparison was made including only five measured specimens per locality for morphologically close-related species, namely *O. amblus*, *O. spheniscus* and *Ornithodoros* cf. *O. yunker*.

6.2.3. Molecular tools

Genetic identification of the tick species was employed to corroborate morphologic diagnoses. For this purpose, DNA extraction using the Guanidine Isothiocyanate technique (Sangioni et al. 2005) was individually performed with four pools of three and five pools of ten laboratory-reared larvae, 30 egg-laying females, two males, and seven nymphs in order to amplify a fragment of the tick mitochondrial 16S rRNA gene by conventional PCR using primers shown in Table 1. Subsequently, two pools of two and one of ten larvae, three nymphs, one male and 15 egg-laying

females were independently submitted to different PCR protocols. Partial fragments of the tick mitochondrial cytochrome oxidase c subunit 1 (COI) gene and 16S rRNA, *GroEL*, and *PyrG* genes from *Coxiella*-like endosymbionts were targeted with primers listed in Table 1. All positive PCR products of expected size were purified with ExoSAP-IT (USB Corporation, Cleveland, USA) and further sequenced using the BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Austin, USA) in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, ABI 3500 Genetic Analyzer, Foster City, CA) with the same primers selected for PCR. Generated sequences were assembled, and primer-trimmed with Geneious R9 software (Kearse et al. 2012), and then submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) in order to infer closest similarities with other published organisms available in GenBank (Altschul et al. 1990)

Table 1. Primers used in the current study.

Gene	Function	Primer	Nucleotide sequence (3'-5')	Fragment size	Reference
Tick					
16S rRNA	Mitochondrial minor ribosomal subunit	16S+1F 16S-1R	CCGGTCTGAACTCAGATCAAGT GCTCAATGATTTTTTAAATTGCTGT	PCR: 16S+1/16S-1: 460-bp	(Mangold et al. 1998)
COI	Cytochrome oxidase subunit c	C1-J-1718 C1-N-2329	GGAGGATTTGGAAATTGATTAGTTCC ACTGTAAATATATGATGAGCTCA	PCR: C1-J-1718/C1-N-2329: 611-bp	(Simon et al. 1994)
Coxiella					
16S rRNA	Bacterial minor ribosomal subunit	Cox16SF1 Cox16SR2	CGTAGGAATCTACCTTRTAGWGG GCCTACCCGCTTCTGGTACAATT	1 st PCR: Cox16SF1/Cox16SR2 : 1321 – 1416-bp	(Duron et al. 2014)
		Cox16SF2 Cox16SR1	TGAGAACTAGCTGTTGGRRAGT ACTYYCCAACAGCTAGTTCTCA	Nested a) Cox16SF2/Cox16SR2: 624 – 627-bp Nested b) Cox16SF1/Cox16SR1: 719 – 813-bp	
<i>GroEL</i>	Chaperonine gene	CoxGrF1 CoxGrR2 CoxGrF2 CoxGrR1	TTTGAAAAYATGGGCGCKCAAATGGT CGRTCRCCAAARCCAGGTGC GAAGTGGCTTCGCRTACWTCAGACG CCAAARCCAGGTGCTTTYAC	1st PCR: CoxGrF1/CoxGrR2: 655-bp Nested: CoxGrF2/CoxGrR1: 619-bp	(Duron et al. 2014)
<i>PyrG</i>	CTP synthase gene	pyrGF pyrGR	TTATTTACCAACGTTCTGAGCCG TTTATCCCGAGCAAATTCAATTATGG	PCR: pyrGF/ pyrGR: 504-bp	(Reeves et al. 2006)

6.2.4. Phylogenetic analyses

6.2.4.1. Ticks

In order to maximize the scope of the phylogenetic analysis, and to present a phylogeny for the Argasidae family, representative tick mitochondrial 16S rDNA sequences of the species identified in this study were aligned with 78 *Ornithodoros*, ten *Argas*, four *Antricola* and two *Nothoaspis* homologous sequences available in GenBank using Clustal X (Thompson et al. 1997), and manually adjusted using GeneDoc (Nicholas et al. 1997). The obtained alignment was used to perform two phylogenetic analyses. A Maximum Parsimony (MP) tree was constructed using PAUP version 4.0b10 (Swofford 2002) with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. Subsequently, a Bayesian analysis (BA) was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences from *Ixodes holocyclus* Neumann 1899 and *Ixodes uriae* White 1852 were used as out-group. Accessions numbers of all sequences are shown in the phylogenetic tree.

Following the topology of previously constructed phylogenies, and in order to gain an accurate definition on the position of all *O. capensis* s. l. sequences generated in this study, a third phylogenetic tree was constructed with 22 other *Ornithodoros* species. The alignment of these sequences was constructed with the same software above stated, and the tree was inferred by means of a Bayesian approach with the same software and specifications as above as well. Distance values within and between clades were calculated for this phylogenetic tree.

Congruent phylogenies between ticks and their *Coxiella*-like endosymbionts were evaluated by the construction of separated phylogenetic trees. For this purpose, sequences of tick mitochondrial 16S rRNA and COI genes from

specimens positive to all three *Coxiella*-like genes, were individually aligned with the CLUSTAL W algorithm (Thompson et al. 1994) implemented in Geneious R9 (Kearse et al. 2012), manually concatenated into a 1045-bp fragment, and subsequently submitted to a Bayesian analysis using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences of *Ornithodoros rostratus* Aragão 1911 (DQ295780; KC769592) were used as out-group.

6.2.4.2. *Coxiella*-like endosymbionts

Independent alignments for each *Coxiella*-like gene using sequences obtained in the current study, were initially constructed with the CLUSTAL W algorithm (Thompson et al. 1994) implemented in Geneious R9 (Kearse et al. 2012). Subsequently, the alignments of 16S rRNA, *GroEL*, and, *PyrG* genes were manually concatenated into a 2457-bp fragment, and submitted to a Bayesian analysis using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences of the *Coxiella*-like endosymbionts associated to *O. rostratus* (KP994794, KP985468, JN887880) rooted the tree. Subsequently, phylogenetic analyses of concatenated tick mitochondrial 16S rRNA/COI and *Coxiella*-like 16S rRNA/*GroEL*/*PyrG* matrixes were visually contrasted by mounting a tanglegram.

6.3. RESULTS

6.3.1. Morphology of adult stages

By a morphological examination of adult stages (males and females), four morphotypes were recognized and classified into species level by the following combination of morphological characters: 1) *Ornithodoros amblus*: body outline parallel laterally, anteriorly and posteriorly rounded; dorsal mammillae smooth and regularly spaced with small interleaved setae (Clifford et al. 1980); dorsal disks with different sizes and conformation as follows: anterior centrolateral slightly smaller than central pairs; anteromedian disk absent; anterolateral line with three group disks (I, II and III + IV); anterior intermedian disk septated but not divided, central intermedian not divided; posterior intermedian slightly smaller than central intermedian; median disk not defined, fused with the posteromedian file into small disks; posteromedian file reaching the posterior 1/3 of the body length; posterolateral file composed by many small disks with a diffused pattern (Figure 3A); 2) *Ornithodoros spheniscus*: body outline parallel laterally, posteriorly rounded, and anteriorly converging to a small blunt apex; dorsal mammillae smooth with irregular outlines, larger in the margins, with very small interleaved setae (Hoogstraal et al. 1985); dorsal disks with different sizes and conformation as follows: anterior centrolateral evidently small than anterior central pair; anteromedian disk present; anterolateral line with four group of disks (I, II, III and IV); anterior intermedian divided into two separated groups of disks, central intermedian not divided; posterior intermedian slightly smaller than central intermedian, composed by a group of small disks; median disk defined and separated from the posteromedian file; posteromedian file reaching almost posterior 1/3 of the body length; small disk present between median disk and posteromedian file; posterolateral file with a defined group of large disks in the first portion, then small disks posteriorly (Figures 3B); 3) *Ornithodoros* cf. *O. yunker*: body outline sub-parallel laterally, posteriorly rounded, and anteriorly converging to a broadly pointed apex; dorsal mammillae large, not uniformly sized and deeply separated (Keirans et al. 1984); conspicuous

setae arising marginally or near the base of some mammillae, evidently observed in the margins; dorsal disks with different sizes and conformation as follows: anterior centrolateral and central pairs almost equal in size; anteromedian disk absent; anterolateral line with four defined but closely disposed disks (I, II, III and IV); anterior intermedian divided in two groups but not separated, central intermedian and posterior intermedian disks not divided; posterior intermedian smaller than central intermedian; median disk not defined, fused with the posteromedian file into small disks; posteromedian line reaching the posterior 1/3 of the body length; posterolateral file with a barely defined larger group of disks in the first portion, then smaller disks posteriorly (Figures 3C); and 4) *Ornithodoros capensis*: body outline sub-parallel laterally, posteriorly rounded, and anteriorly converging to a broadly pointed apex; dorsal mammillae large, uniform in size, with very small interleaved setae; dorsal disk chiefly uniform in sizes, made of single unit, and conformation as follows: anterior centrolateral and central pairs almost equal in size; anteromedian disk absent; anterolateral line with four defined closely disposed disks (I, II, III and IV); anterior and central intermedian divided in two disks; anterior intermedian slightly separated, central intermedian not separated; posterior intermedian disks not divided; median disk defined and separated from the posteromedian file (a small disk present between median disk and posteromedian file in some specimens); posterolateral file with three defined groups of disks (Figure 3D and E).

The above external characters were identical among males and females of each of the four tick species.

Figure 3. Draw of body outline and dorsal disk topology of the adult stages of *Ornithodoros* identified in this study. (A) *Ornithodoros amblus*, (B) *Ornithodoros spheniscus*, (C) *Ornithodoros* cf. *O. yunker*, (D) *Ornithodoros capensis* from Tocopilla (Northern Chile), and (E) *Ornithodoros capensis* from São Pedro and São Paulo Archipelago (Atlantic Ocean, Brazil). Abbreviations: AI, anterior intermedian; CI, central intermedian. Scale bar is equivalent to 1 mm.

6.3.2. Morphology of larvae

Overall, ten larvae from each of the following species per locality were measured: 1) *Ornithodoros amblus*: Pan de Azúcar Island, Santa María Island, Paracas, Trujillo, and Foca Island; 2) *Ornithodoros spheniscus*: The Death Islet, Pájaros Island, Chañaral Island, Pan de Azúcar Island, and Foca Island; 3) *Ornithodoros* cf. *O. yunker*i: Taltal, Santa María Island, Tocopilla, Piedra Blanca, Chala, and San Juan de Marcona; and 4) *Ornithodoros capensis*: Isla Foca. Additionally, six larvae of *O. capensis* were measured from one female collected in São Pedro and São Paulo Archipelago.

Morphological characters of larvae obtained from diagnosed females matched the respective descriptions for *O. amblus*, *O. spheniscus* (Figure 4, Table 2) and *O. capensis* (Figure 5, Table 2). However, following the original description of *O. yunker*i (Keirans et al. 1984), a slight but noteworthy difference with *Ornithodoros* cf. *O. yunker*i from this study was observed in the hypostome: the larva of *O. yunker*i has four files of denticles, in contrast to three in *Ornithodoros* cf. *O. yunker*i larvae (Figure 4, Table 2). Slide mounted larvae were deposited at the “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” under the following accession numbers: CNC-3608, -3609, -3610, -3611, and 3612.

Table 2. Measurements (μm) of morphological characters from unengorged larval stages examined in this study.

Character	<i>O. amblus</i>	<i>O. spheniscus</i>	<i>Ornithodoros</i> cf. <i>O. yunker</i>	<i>O. capensis</i> (Pacific Ocean, Peru)	<i>O. capensis</i> (Atlantic Ocean, Brazil)
Body length including capitulum	825 $\pm$ 62 (725-960)	753 $\pm$ 45 (667-833)	695 $\pm$ 22 (652-744)	611 $\pm$ 17 (585-640)	574 $\pm$ 26 (518-621)
Body length not including capitulum	573 $\pm$ 46 (491-686)	521 $\pm$ 40 (436-588)	474 $\pm$ 30 (417-526)	427 $\pm$ 21 (395-469)	406 $\pm$ 18 (380-438)
Body width	526 $\pm$ 64 (426-676)	499 $\pm$ 35 (441-593)	460 $\pm$ 29 (395-509)	380 $\pm$ 19 (360-421)	376 $\pm$ 26 (329-426)
Dorsal plate: length	265 $\pm$ 19 (208-304)	231 $\pm$ 11 (196-259)	260 $\pm$ 9 (235-278)	204 $\pm$ 9 (185-219)	183 $\pm$ 11 (153-199)
Dorsal plate: width	195 $\pm$ 21 (156-251)	196 $\pm$ 13 (167-222)	179 $\pm$ 10 (165-215)	149 $\pm$ 11 (130-160)	136 $\pm$ 9 (122-149)
Dorsal setae: total pairs	15	15	15	24 (24-25)	25 (24-25)
Dorsal setae: dorsolateral pairs	11	11	11	20 (20-21)	21 (20-21)
Dorsal setae: central pairs	4	4	4	4	4
Dorsal anterolateral setae: AI 1	122 $\pm$ 8 (100-139)	116 $\pm$ 11 (96-144)	119 $\pm$ 7 (102-140)	102 $\pm$ 6 (95-113)	108 $\pm$ 6 (97-118)
Dorsal anterolateral setae: AI 2	130 $\pm$ 8 (107-146)	120 $\pm$ 9 (99-140)	129 $\pm$ 10 (111-185)	104 $\pm$ 6 (97-114)	111 $\pm$ 8 (96-121)
Dorsal anterolateral setae: AI 3	135 $\pm$ 11 (99-161)	117 $\pm$ 11 (86-146)	143 $\pm$ 9 (115-165)	108 $\pm$ 6 (101-115)	118 $\pm$ 8 (93-130)
Dorsal anterolateral setae: AI 4	124 $\pm$ 9 (110-146)	116 $\pm$ 9 (94-144)	125 $\pm$ 8 (104-150)	101 $\pm$ 6 (91-113)	110 $\pm$ 7 (100-120)
Dorsal anterolateral setae: AI 5	136 $\pm$ 11 (115-163)	112 $\pm$ 14 (83-146)	137 $\pm$ 9 (112-158)	102 $\pm$ 7 (94-119)	113 $\pm$ 6 (102-123)
Dorsal anterolateral setae: AI 6	119 $\pm$ 8 (103-134)	122 $\pm$ 10 (78-93)	120 $\pm$ 8 (103-139)	100 $\pm$ 9 (91-115)	108 $\pm$ 8 (97-120)
Dorsal anterolateral setae: AI 7	139 $\pm$ 10 (57-105)	115 $\pm$ 14 (83-141)	141 $\pm$ 15 (48-166)	104 $\pm$ 6 (95-114)	111 $\pm$ 9 (97-127)
Dorsal posterolateral setae: PI 1	102 $\pm$ 9 (80-122)	107 $\pm$ 11 (84-137)	100 $\pm$ 11 (38-122)	78 $\pm$ 3 (71-81)	86 $\pm$ 7 (71-99)
Dorsal posterolateral setae: PI 2	99 $\pm$ 8 (85-117)	111 $\pm$ 10 (78-127)	99 $\pm$ 10 (39-118)	79 $\pm$ 8 (70-96)	85 $\pm$ 8 (73-98)
Dorsal posterolateral setae: PI 3	95 $\pm$ 9 (71-116)	114 $\pm$ 13 (75-142)	95 $\pm$ 10 (36-114)	85 $\pm$ 5 (77-94)	87 $\pm$ 6 (80-102)
Dorsal posterolateral setae: PI 4	83 $\pm$ 10 (57-105)	100 $\pm$ 14 (70-135)	90 $\pm$ 7 (74-102)	79 $\pm$ 8 (70-96)	86 $\pm$ 7 (71-97)
Dorsal posterolateral setae: PI 5	Absent	Absent	Absent	85 $\pm$ 5 (72-97)	87 $\pm$ 9 (60-100)
Dorsal posterolateral setae: PI 6	Absent	Absent	Absent	84 $\pm$ 6 (72-92)	89 $\pm$ 7 (79-105)
Dorsal posterolateral setae: PI 7	Absent	Absent	Absent	79 $\pm$ 10 (60-91)	87 $\pm$ 7 (72-96)
Dorsal posterolateral setae: PI 8	Absent	Absent	Absent	79 $\pm$ 8 (72-100)	85 $\pm$ 6 (75-95)
Dorsal posterolateral setae: PI 9	Absent	Absent	Absent	73 $\pm$ 8 (63-87)	87 $\pm$ 8 (75-101)
Dorsal posterolateral setae: PI 10	Absent	Absent	Absent	79 $\pm$ 11 (58-92)	84 $\pm$ 7 (69-94)
Dorsal posterolateral setae: PI 11	Absent	Absent	Absent	74 $\pm$ 10 (59-89)	82 $\pm$ 8 (64-95)

Table 2. (Continued).

Character	<i>O. amblus</i>	<i>O. spheniscus</i>	<i>Ornithodoros</i> cf. <i>O. yunkeri</i>	<i>O. capensis</i> (Pacific Ocean, Peru)	<i>O. capensis</i> (Atlantic Ocean, Brazil)
Dorsal posterolateral setae: Pl 12	Absent	Absent	Absent	76 ± 7 (66-89)	81 ± 8 (65-91)
Central setae: C1	131 ± 9 (107-151)	108 ± 10 (85-134)	123 ± 10 (90-146)	98 ± 6 (89-110)	109 ± 10 (85-123)
Central setae: C2	103 ± 10 (79-122)	83 ± 13 (61-117)	99 ± 7 (79-115)	78 ± 7 (66-89)	84 ± 7 (71-98)
Central setae: C3	91 ± 7 (74-105)	84 ± 13 (60-110)	93 ± 8 (74-110)	81 ± 5 (74-89)	88 ± 5 (78-97)
Central setae: C4	88 ± 7 (73-109)	100 ± 13 (74-137)	87 ± 7 (72-104)	80 ± 8 (63-93)	83 ± 8 (65-93)
Sternal setae: St 1	86 ± 6 (71-102)	76 ± 7 (64-90)	75 ± 4 (63-83)	61 ± 6 (49-70)	62 ± 4 (53-68)
Sternal setae: St 2	89 ± 6 (75-102)	73 ± 6 (56-84)	72 ± 4 (61-83)	61 ± 4 (52-67)	61 ± 6 (51-71)
Sternal setae: St 3	93 ± 8 (75-110)	78 ± 7 (64-93)	75 ± 4 (65-87)	60 ± 7 (46-70)	61 ± 7 (48-71)
Circumanal setae: Ca1	76 ± 9 (58-112)	66 ± 8 (49-82)	63 ± 6 (52-74)	60 ± 7 (46-67)	62 ± 9 (48-77)
Circumanal setae: Ca2	95 ± 8 (78-112)	108 ± 12 (69-132)	87 ± 7 (69-110)	81 ± 5 (82-99)	87 ± 8 (76-100)
Circumanal setae: Ca3	113 ± 9 (96-138)	131 ± 12 (99-163)	106 ± 8 (78-124)	91 ± 5 (82-99)	96 ± 7 (84-108)
Posteromedian: Pms	Absent	Absent	Absent	67 ± 10 (55-77)	70 ± 5 (61-77)
Postcoxal setae: Pc	62 ± 6 (49-80)	72 ± 12 (42-95)	63 ± 6 (51-73)	58 ± 4 (52-63)	62 ± 7 (48-72)
Length of basis capituli to Ph1	101 ± 11 (83-127)	101 ± 11 (75-124)	89 ± 11 (54-110)	84 ± 8 (67-94)	74 ± 7 (63-91)
Length of basis capituli to insertion of hypostome	122 ± 15 (92-146)	118 ± 17 (86-156)	104 ± 15 (65-138)	105 ± 7 (94-114)	89 ± 10 (73-106)
Length of capituli	326 ± 22 (271-363)	311 ± 23 (266-353)	274 ± 16 (233-304)	261 ± 10 (244-274)	233 ± 11 (212-254)
Width of basis capituli	196 ± 15 (167-227)	185 ± 14 (150-212)	180 ± 13 (152-208)	157 ± 9 (143-172)	146 ± 12 (130-173)
Posthypostomal setae Ph1	9 ± 2 (6-13)	8 ± 2 (3-12)	9 ± 1 (6-14)	-	-
Posthypostomal setae Ph2	24 ± 4 (15-35)	24 ± 4 (17-31)	20 ± 3 (11-30)	14 ± 3 (12-20)	17 ± 4 (8-26)
Distance Ph1 - Ph1	28 ± 3 (21-34)	27 ± 2 (22-33)	31 ± 9 (17-59)	21 ± 3 (17-28)	18 ± 3 (15-26)
Distance Ph2 - Ph2	94 ± 6 (79-112)	95 ± 7 (78-110)	83 ± 5 (72-95)	93 ± 4 (85-97)	85 ± 3 (78-90)
Palpal length	243 ± 15 (213-281)	238 ± 14 (207-261)	207 ± 10 (185-228)	198 ± 8 (184-211)	178 ± 6 (169-193)
Length article I	51 ± 5 (41-63)	51 ± 5 (40-66)	40 ± 3 (32-49)	41 ± 3 (37-47)	37 ± 4 (32-44)
Length article II	79 ± 5 (70-89)	77 ± 6 (40-66)	68 ± 4 (57-78)	62 ± 3 (56-67)	57 ± 3 (52-62)
Length article III	78 ± 4 (70-85)	73 ± 6 (54-86)	64 ± 4 (57-78)	61 ± 4 (57-67)	57 ± 4 (48-65)
Length article IV	55 ± 5 (44-64)	55 ± 5 (44-66)	51 ± 5 (40-65)	46 ± 4 (38-52)	42 ± 3 (38-48)

Table 2. (Continued).

Character	<i>O. amblus</i>	<i>O. spheniscus</i>	<i>Ornithodoros</i> cf. <i>O. yunkeri</i>	<i>O. capensis</i> (Pacific Ocean, Peru)	<i>O. capensis</i> (Atlantic Ocean, Brazil)
Width article I	33 ± 2 (28-37)	32 ± 3 (24-38)	29 ± 2 (25-33)	27 ± 2 (24-31)	25 ± 2 (22-28)
Width article II	35 ± 2 (31-39)	31 ± 2 (27-36)	32 ± 2 (29-39)	26 ± 1 (25-29)	25 ± 2 (23-28)
Width article III	35 ± 3 (27-39)	35 ± 3 (28-39)	33 ± 2 (24-37)	31 ± 1 (29-33)	28 ± 2 (25-31)
Width article IV	25 ± 2 (20-32)	24 ± 1 (21-27)	22 ± 2 (11-28)	20 ± 1 (18-22)	20 ± 2 (16-22)
Hypostome: length measured to point to Ph1	224 ± 15 (191-259)	210 ± 12 (188-232)	185 ± 8 (164-205)	178 ± 5 (170-190)	161 ± 5 (155-170)
Hypostome: length measured to the inferior toothed portion	207 ± 11 (180-234)	191 ± 9 (171-205)	175 ± 6 (162-188)	156 ± 4 (150-161)	147 ± 4 (140-153)
Hypostome: base width	61 ± 10 (47-88)	51 ± 8 (41-70)	48 ± 7 (36-61)	57 ± 4 (50-63)	47 ± 3 (42-53)
Hypostome: middle width	64 ± 8 (53-85)	55 ± 7 (46-83)	56 ± 7 (42-69)	63 ± 4 (57-70)	52 ± 4 (47-59)
Apical dental formula	4	4	3	5	5
Median dental formula	3 (3-4)	2 (2-3)	2 (2-3)	4	3 (3-4)
Basal dental formula	2	2	2	2	2
Denticles in hypostomal row 1	18 ± 2 (15-21)	19 ± 1 (16-21)	18 ± 1 (15-20)	13 ± 1 (12-14)	16 ± 2 (13-18)
Denticles in Hypostomal row 2	17 ± 1 (14-19)	16 ± 1 (14-18)	17 ± 1 (14-19)	12 ± 1 (11-13)	14 ± 1 (12-15)
Denticles in Hypostomal row 3	11 ± 1 (8-13)	9 ± 1 (7-11)	10 ± 1 (8-12)	8 ± 1 (7-10)	10 ± 1 (8-11)
Denticles in Hypostomal row 4	8 ± 1 (5-11)	5 ± 1 (3-7)	-	6 ± 1 (5-7)	7 ± 1 (6-8)
Denticles in Hypostomal row 5	-	-	-	5 ± 1 (4-6)	5 ± 1 (4-6)
Tarsus I: length	224 ± 14 (188-248)	234 ± 17 (191-268)	207 ± 10 (175-223)	209 ± 6 (202-221)	186 ± 6 (174-196)
Tarsus I: width	66 ± 5 (58-75)	64 ± 5 (54-81)	62 ± 5 (51-73)	55 ± 2 (52-58)	54 ± 4 (48-62)

Figure 4. Micrographs of *O. amblus*, *O. spheniscus*, and *Ornithodoros* cf. *O. yunkerii*. *Ornithodoros amblus*: (A) Dorsal view, (B) dorsal plate, and (C) hypostome; *Ornithodoros spheniscus*: (D) Dorsal view, (E) dorsal plate, and (F) hypostome; *Ornithodoros* cf. *O. yunkerii*: (G) Dorsal view, (H) dorsal plate, and (I) hypostome.

Figure 5. Micrographs of *O. capensis* from Foca Island. (A) Dorsal view, (B) dorsal plate, and (C) hypostome.

6.3.3. PCA

The results of the PCA for the specific cohorts of larvae submitted to comparison showed relatively low values of total variance as follows: 29.35% (*Ornithodoros* cf. *O. yunkeri*); 42.23% (*O. capensis*); 44.57% (*O. amblus*), and 52.56% (*O. spheniscus*). In each independent analysis, the first principal component explained most of the observed variance, and was almost fully loaded by the following characters: 1) *Ornithodoros amblus* (35.74%): body length, length of capituli and length of hypostome; 2) *Ornithodoros* cf. *O. yunkeri* (19.17%): length of basis capituli and palpal length; 3) *Ornithodoros spheniscus* (42.41%): dorsal anterolateral setae and palpal lengths; and 4) *Ornithodoros capensis* (29.52%): dorsal anterolateral setae and capituli lengths. On the other hand, the rest of the variance explained by the second component included the following heavier characters: 1) *Ornithodoros amblus* (8.83%): palpi and dorsolateral setae lengths; 2) *Ornithodoros* cf. *O. yunkeri* (10.17%): hypostome length and body with; 3) *Ornithodoros spheniscus* (10.16%): basis capituli and posthypostomal setae lengths; and 4) *Ornithodoros capensis* (22.71%): dorsal posterolateral and sternal setae lengths (Figure 6A, B, C, and D).

The analysis performed for larvae of *O. amblus*, *O. spheniscus* and *Ornithodoros* cf. *O. yunkeri* showed an incomplete separation of each species, reflected by a total variance of 49.02%, and mostly explained by the first principal component (42.90%), and it was almost fully loaded with the body length (including capitulum), length of capituli, palpi and hypostome (measured from the apex to point to Ph1 setae). The second component explained a 26.12% of the variance the dorsal plate, dorsal anterolateral, and central setae lengths were the heavier characters (Figure 7).

Figure 6. Principal component analyses performed with the cohorts of laboratory-reared larvae obtained from females of four species: (A) *Ornithodoros ambulus*, (B) *Ornithodoros spheiscus*, (C) *Ornithodoros* cf. *O. yunker*, and (D) *Ornithodoros capensis*. Localities of collection are indicated for each cluster of points. Abbreviations: F1, first component axis; F2, second component axis.

Figure 7. Principal component analysis performed for larvae of *O. amblus* (bluish points), *O. spheniscus* (yellowish points), and *Ornithodoros* cf. *O. yunkeri* (reddish points). Localities of collection are indicated for each cluster of points. Abbreviations: F1, first component axis; F2, second component axis.

6.3.4. PCR

Overall at least one species from each locality yielded positive amplicons for the performed PCRs (Table 3). Obtained fragments of 16S mitochondrial rRNA varied in size (primers excluded): *O. amblus* 420 - 421-bp, *O. spheniscus* 418 – 420-bp, *Ornithodoros* cf. *O. yunker* 419 – 421-bp, and *O. capensis* 422 – 426-bp. Different haplotypes were obtained in each species as follows: 1) *Ornithodoros amblus*: four haplotypes, one from Trujillo and Punta Picata (Peru); one from Tanaka (Peru), and Taltal (Chile); one from Pan de Azúcar Island (Chile); and one from Foca Island, Tanaka, San Juan de Marcona, Paracas (Peru), Santa María Island, Pájaros Island, and Pan de Azúcar Island (Chile); 2) *Ornithodoros spheniscus*: five haplotypes, three from The Death Island; one from Chañaral Island; and one from Pan de Azúcar Island (Chile); 3) *Ornithodoros* cf. *O. yunker*: three haplotypes, one from San Juan de Marcona, Chala, Tanaka (Peru), Caleta Vitor and Taltal (Chile); one from Piedra Blanca and Punta Picata (Peru), Caleta Vitor, Santa María Island, and Taltal (Chile); and one from Chala, Tanaka, Punta Picata (Peru), Tocopilla, Pájaros Island and Taltal (Chile); and 4) *Ornithodoros capensis*: three haplotypes, one from Foca Island (Peru); one from Queimada Grande and São Pedro and São Paulo Archipelago (Atlantic Ocean, Brazil); and one from San Juan de Marcona (Peru), Tocopilla, Santa María Island, and Pájaros Island (Chile). Although *Coxiella*-like endosymbionts were amplified in two pools of three larvae, three nymphs, and one male and 20 females, only in 15 females and two nymphs the complete array of targeted genes (16S rRNA, *GroEL*, and *Pyrg*) were successfully amplified and sequenced.

Table 3. Ticks submitted to DNA extraction, and results of PCR-targeted genes.

Tick species Locality	Code	Stage submitted to DNA extraction	Tick		Coxiella-like endosymbiont		
			16S	COI	16S	GroEL	Pyrg
<i>Ornithodoros ambulus</i>							
Pájaros Island (Chile)	Oa37	1F	+		+	+	
	Oa73	3L*	+		+	+	
Pan de Azúcar Island (Chile)	Oa47	1F	+	+	+	+	+
	Oa69	1N	+		+	+	
Taltal (Chile)	Oa99	10L*	+				
Santa María Island (Chile)	Oa80	1F	+		+		
	Oa98	10L*	+				
Picata (Peru)	Oa63	1F	+	+	+	+	+
Tanaka (Peru)	Oa66	1M	+		+		
	Oa88	1N	+				
San Juan de Marcona (Peru)	Oa59	1N	+				
Paracas (Peru)	Oa93	1F	+				
Trujillo (Peru)	Oa92	1F	+	+			
Foca Island (Peru)	Oa94	1F	+	+			
<i>Ornithodoros capensis</i>							
Pájaros Island (Chile)	Oc90	1F	+	+	+	+	+
Santa María Island (Chile)	Oc27	1F	+	+	+	+	+
Tocopilla (Chile)	Oc91	1F	+				
San Juan de Marcona (Peru)	Oc58	1F	+				
Foca Island (Peru)	Oc97	1F	+	+			
São Pedro e São Paulo (Brazil)	Oc76	1F	+	+	+	+	+
Queimada Grande Island (Brazil)	Oc140	1F**	+	+	+	+	+
<i>Ornithodoros sphegniscus</i>							
The Dead Islet (Chile)	Os102	1F	+				
	Os86a	10L*	+			+	
	Os34	1F	+	+	+	+	+
Pájaros Island (Chile)	Os71	3L*	+				
	Os28	1F	+	+	+	+	+
Chañaral Island (Chile)	Os74	1F	+	+	+	+	+
Pan de Azúcar Island (Chile)	Os2	1F	+				
	Os1	1F	+	+	+	+	+
Foca Island	Os103	1F					
<i>Ornithodoros cf. O. yunkerii</i>							
Pájaros Island (Chile)	Ocfy77	1M	+				
Taltal (Chile)	Ocfy83	1F	+	+	+	+	+
	Ocfy101	10L*	+				
Santa María Island (Chile)	Ocfy100	10L*	+				
	Ocfy81	1F	+				
Tocopilla (Chile)	Ocfy67	1F	+	+	+	+	+
Caleta Vitor (Chile)	Ocfy78	1N	+				
	Ocfy79	1N	+				
Punta Picata (Peru)	Ocfy64	1F	+				
	Ocfy26	1N	+	+	+	+	+
Piedra Blanca (Peru)	Ocfy61	3L*	+				
	Ocfy19	1F	+	+	+	+	+
Chala (Peru)	Ocfy23	1F	+	+	+	+	+
	Ocfy62	3L*	+		+		
Tanaka (Peru)	Ocfy65	1F	+		+		
	Ocfy86b	1F	+		+		
San Juan de Marcona (Peru)	Ocfy60	1N	+		+		+
	Ocfy20	1F	+	+	+	+	+

*Pools of larvae obtained from a unique female cohort when corresponding females died after oviposition.

**Amplification was performed with DNA already extracted in a previous study (Muñoz-Leal et al. 2017).

6.3.5. Phylogenetic analyses

6.3.5.1. Ticks

The phylogenetic analysis performed with representative sequences obtained in this study, in conjunct with all the sequences of *Ornithodoros* spp. retrieved from GenBank, resulted into a phylogenetic tree with logic topology, clearly separating the *Argas* genus from *Ornithodoros* representatives. On the one hand, sequences from *O. amblus*, *O. spheniscus* and *Ornithodoros* cf. *O. yunker*, and on the other hand *O. capensis* formed two independent monophyletic groups. Under a relatively high bootstrap support and Bayesian posterior probability, the clade conformed by sequences of *O. capensis* from different localities, and *O. sawaii* from Korea, appears paraphyletic with respect to other *Ornithodoros* belonging to the *O. capensis* group. *Ornithodoros saraivai* Muñoz-Leal & Labruna 2017 intercalates its phylogenetic position between the *O. capensis*/*O. sawaii* clade, and a group conformed by *O. maritimus* and other unidentified species of the complex labeled as “*O. capensis*” in GenBank. Moreover, *Ornithodoros microlophi* Venzal, Nava & González-Acuña 2013, appears basal to the group composed by *O. amblus*, *O. spheniscus*, *Ornithodoros* cf. *O. yunker*, *O. denmarki*, and an unidentified *Ornithodoros* sp. from Galapagos Islands (Figure 8).

In a second phylogenetic tree using all the sequences generated in this study with sequences of other representatives of the *O. capensis* group, plus *O. microlophi* and *O. saraivai*, the paraphyly of the group is still maintained, though not so well supported. Still, clear monophyletic groups are conformed by all the identified species. If linking these groups with their geographic distribution, the four identified species overlap its occurrence across the shores of Chile and Peru (Figure 9). Four delimited groups conform *O. capensis* s. l.: 1) a group formed by *O. capensis* s. s. and *O. sawaii*, with distribution in the Atlantic, Indian and Pacific Oceans; 2) a group formed by *O. maritimus* and other unidentified species, with distribution in the Mediterranean Sea and Atlantic Ocean; 3) and a group of Neotropical (*O. amblus*, *O. spheniscus*, *Ornithodoros* cf. *O. yunker* and

unidentified species from Galapagos Islands) and Nearctic species (*O. denmarki*) (Figure 9). Genetic distances calculated for the generated Bayesian tree were variable, but overall did not exceed 2.9% within any of the specific clades for different representatives of *O. capensis* group. Remarkably, a close genetic relatedness (1.8 – 2.1%) was observed for *O. amblus* and the sequences of the *Ornithodoros* sp. from Galapagos (JQ824300, JQ824301, JQ824302). In turn, this last unidentified species was 3.2 – 3.5% distanced from *Ornithodoros* cf. *O. yunker* (Table 4).

Figure 8. Bayesian and Maximum Parsimony trees of the Argasidae family. Sequences obtained in this study are highlighted in bold.

Figure 9. Bayesian phylogenetic tree of *O. capensis* s. l. The hypothetical distribution of the four species of *O. capensis* s. l. analyzed in this study is marked with bold lines across Peruvian and Chilean seaboard, and two insular territories from Brazil. Light blue, *O. amblus*; green, *O. capensis*; yellow, *O. spheiscus*, and red, *Ornithodoros* cf. *O. yunker*. Although *O. spheiscus* was not collected in the northern shores of Peru, the dotted yellow line in the map extends until the northernmost locality of occurrence for this species (Punta Blanca, Piura Departament) stated in its original description (Hoogstraal et al. 1985).

Table 4. Bayesian distance values calculated for different representatives of *O. capensis* s. l. of Figure 9 phylogenetic tree. Intraspecific distance is highlighted in bold; the rest of the values correspond to the distances between clades.

[illegible]

6.3.5.2. *Coxiella*-like endosymbionts and ticks

Coxiella-like endosymbionts were directly associated with their respective ticks, though both phylogenetic trees were different in their topologies. *Ornithodoros spheniscus* appears as a basal group within the clade conformed by *O. amblus* and *Ornithodoros* cf. *O. yunker*, and *O. capensis* stays out of this group in the mitochondrial 16S rRNA/COI phylogeny; whether in the *Coxiella* phylogeny, *O. spheniscus* and *O. capensis* switch their phylogenetic positions with each other (Figure 10).

Figure 10. Tanglegram confronting Bayesian inferred evolutionary histories of ticks analyzed in this study, and their associated *Coxiella*-like endosymbionts.

6.4. DISCUSSION

6.4.1. Morphology of adult stages

Hoogstraal et al. (1986) defined the main morphological characters to separate adult stages of *O. amblus*, *O. spheniscus* and *O. yunker*, making allusion to the format and disposition of mammillae in the dorsum, and to the ultrastructure of disks in each species. Although valuable, these characters are somewhat difficult to observe since a lot of intermediate forms can also occur within a sole species, and because an explicit definition on different format of mammillae, and kinds of disk surfaces, has not been properly published in order to correctly recognize when those discrete characters can be considered different (Klompen and Oliver 1993). Moreover, in the majority of the species, an accurate observation of these traits needs the assistance of scanning electron micrographs, which for several studies are not viable, and expensive to perform. In an attempt to find useful morphological traits to separate adult stages of seabird soft ticks, in this study we used the topology and macro anatomy of dorsal disks. By this approach, four morphotypes of ticks were clearly recognized, and identified to species level based on previous original descriptions. A striking difference, the presence of anteromedian disk, constitutes a primary character in order to separate *O. spheniscus* from *O. amblus*, *O. capensis* and *Ornithodoros* cf. *O. yunker*, since the last three species lack this structure. Remarkably, if extending this comparison to other representatives of *O. capensis* s. l., *O. spheniscus* is the sole species that has this disk, reassembling more with the topology and disk disposition exhibited by *O. talaje* s. l. species. A secondary character useful to separate the examined species was the position of the median disk, which is defined and separated from the posteromedian line in *O. capensis* and *O. spheniscus*, whereas undefined, in touch or fused with the posteromedian line in the two other species (Figure 3). For instance, Klompen and Oliver (1993) performed the sole phylogenetic analysis combining morphological traits of larvae and adults. These authors included the disposition of dorsal disks as a character set, however only absence or presence, and a radial, partially radial or a random disposition were considered as character states (Klompen and Oliver 1993). Taking into account the concrete morphological differences observed in the dorsal pattern of disks in the current study, it would be interesting to repeat such a phylogenetic

analysis considering the topology of disks herein proposed, extending the observations to homologous characters present in other Ornithodorinae.

Neumann described that *O. talaje* var. *capensis* (i. e. *O. capensis* s. s.) resembled more *O. coniceps* than *O. talaje* by presenting the following characters: 1) thinner and less developed cheeks, not capable to completely cover the hypostome and palps, and not reaching the ventral surface of basis capituli; and 2) longer and more abundant hairs on the movable articles (Neumann 1901, Nuttall et al. 1908). In addition to these differences, by a comparison of dorsal disks, adult stages of *O. capensis* s. l. can also be separated from *O. talaje* s. l. because the former lacks the anteromedian disk. The only exception to this observation corresponds to *O. spheniscus* (Figure 2B), which constitutes an interesting case of future study since, despite adults possess anteromedian disk, larvae possess highly similar morphological traits with *O. amblus* and *O. yunkeri*, and it also forms a monophyletic group with Neotropical taxa of seabird soft ticks.

6.4.2. Morphology of larvae

Overall, two groups of larval morphotypes were easily separated by the combination of the following traits: 1) 11 pairs of dorsolateral setae (seven anterolateral, four posterolateral), four central pairs, absence of posteromedian seta (*O. amblus*, *O. spheniscus*, *Ornithodoros* cf. *O. yunkeri*), and 2) 19-21 pairs of dorsolateral pairs (seven anterolateral, 12-13 posterolateral), four central pairs, and presence of posteromedian seta (*O. capensis*).

While describing *O. yunkeri*, Keirans et al. (1985) stated that the best morphological characters to separate larvae of the *O. capensis* group were the dentition of the hypostome, number of setae, and the size and shape of the dorsal plate. When compared with *O. yunkeri*, larvae of *Ornithodoros* cf. *yunkeri* matches the number of dorsal and ventral setae, and the shape of the dorsal plate. However, larvae of *Ornithodoros* cf. *yunkeri* are larger (0.695 ± 0.022 mm vs. 0.450 in *O. yunkeri*), and lack a fourth file of denticles in the distal portion of the hypostome. Although slight, these traits are constant for larvae from different and distanced localities, suggesting that *Ornithodoros* cf. *O. yunkeri* could correspond to a putative new species. A reliable genetic comparison with *O. yunkeri* s. s. is now needed in order to clarify its taxonomic status.

6.4.3 PCA

PCA performed to intraspecific groups of larvae showed low levels of variability, supporting that even if vastly separated, populations of these ticks maintain a defined phenotype. Interestingly, if combining the data on genetic distance, and PCA total variance for the four species analyzed in this study, *O. amblus* and *Ornithodoros* cf. *yunkeri* show low genetic distances and variance values between their populations, and in turn, *O. spheniscus* and *O. capensis* show overall higher values for both approaches. In this sense, it is reasonable to infer that a degree of genetic isolation could be resulting in slight morphological differences between vastly separated populations of *O. spheniscus* and *O. capensis*. Conversely, low genetic distance within *O. amblus* and *Ornithodoros* cf. *O. yunkeri* populations could explain the occurrence of a less variable larval phenotype.

As for other invertebrate classes, taxonomic classifications of ticks have recurrently pointed the occurrence of morphologically related species (Cooley and Kohls 1945, Walker et al. 2000, Geevarghese and Mishra 2011, Guglielmone et al. 2014). The *O. capensis* group is conformed by a group of seabird ticks sharing morphological affinities in larval and adult stages (Hoogstraal 1985, Hoogstraal et al. 1985). Particularly, although adult stages of *O. amblus*, *O. spheniscus*, and *Ornithodoros* cf. *O. yunkeri* have recognizable differences between them, larvae of all three species have extremely similar phenotypes. These morphological affinities were tested out by the PCA, since relatively low values of total variance were calculated when comparing these three species (Figure 7). Among larval stages, the dimension of the capitulum (total length; palpi and hypostome lengths) explained most of the calculated variance. This anatomical structure is in direct contact with the host skin, and it guarantees a successful attaching during the feeding period. In this sense, slight variability evidenced in the PCA analysis of such a crucial anatomical structure could be evidence of adaptations to different characteristics of host skins. Anyway, a histologic study on skin composition, accompanied by the identification of larvae directly collected upon their hosts, would be informative in order to support this hypothesis.

Larval stages of *Ornithodoros* (*Alectorobius*) species are slow feeding (Oliver 1989), and particularly, for *O. amblus* and *O. spheniscus* it takes 4 – 7 days to fully engorge, and subsequently detach from the host (Clifford et al. 1980, Hoogstraal et

al. 1985). Compared with conspecific fast-feeding nymph and adult stages, that complete their meal in minutes to hours (Clifford et al. 1980, Hoogstraal et al. 1985), larvae of these species spent a considerable amount of time of their lifespan in contact with their hosts. Considering the biological importance of a successful feeding, that ultimately will enable the molting to the following two nymphal instars, adaptation to the avian host appears as a pivotal event in the evolution of seabird *Alectorobius* ticks. In this context, discounting the slight differences in body size and capituli, affinities in the external morphology between different species of bird-associated ticks, and particularly in *O. amblus*, *O. spheniscus* and *Ornithodoros* cf. *O. yunkeri*, could be the result of common selective pressures directly imposed by the feathery on-host milieu.

6.4.4. Phylogenetic analyses

6.4.4.1. Ticks

The phylogenetic analysis of a partial mitochondrial 16S rDNA fragment comprising the majority of the Argasidae species available in GenBank showed that the *O. capensis* group is paraphyletic. Remarkably, the phylogenetic positions of *O. saraivai*, an amphibian-associated soft tick that in a previous phylogenetic analysis formed a monophyletic group with *Ornithodoros faccinii* Barros-Battesti, Landulfo & Luz 2015 in Brazil, and *O. microlophi*, a reptile-associated tick inhabiting northern shores of Chile, divide the *O. capensis* group into three delimited groups: *O. capensis* including sequences from the Atlantic, Indian and Pacific Ocean, plus *O. sawaii*, a group from the Mediterranean Sea, Cabo Verde, Canarian Islands and northern Africa including *O. maritimus* plus other *Ornithodoros* associated to seabird; and a Neotropical-Nearctic array of species composed by *O. amblus*, *O. spheniscus*, *Ornithodoros* cf. *yunkeri*, an *Ornithodoros* sp. from Galapagos, and *O. denmarki*. Remarkably, as showed by previous phylogenetic analyses (Dupraz et al. 2016, Kim et al. 2017, Muñoz-Leal et al. 2017), *O. sawaii* reiteratively clusters within the *O. capensis* clade, a fact that once more tames the classification of this species as a valid taxon within the Argasidae.

Taking into account morphological characters of adults and larvae, the split of *O. capensis* s. l. is not surprising. However, it is important to mention that this

phylogenetic scenario was inferred by the analysis of a partial fragment of the mitochondrial 16S rRNA gene, which is not free of introducing ambiguities in phylogenetic trees due to homoplasy events (Avise et al. 1987). On the other hand, even though the mitochondrial 16S rRNA gene has been widely used to genetically identify ticks, in soft ticks a relatively small amount of the total diversity has yet to be characterized. In this way, the inclusion of new terminals into a still incomplete array of operational taxonomic units might lead to unstable topologies within a phylogenetic reconstruction (Graybeal 1998).

6.4.4.2. Genetic distance

Recently, by means of cross-mating experiments Labruna et al. (2016) demonstrated that a genetic divergence of 3.3% in mitochondrial 16S rDNA sequences between two populations of *Ornithodoros rietcorrei* Labruna, Nava & Venzal 2016 still resulted in reproductive compatibility. This evidence suggests that genetic distances observed within the *O. capensis* clade (2.9%), which is conformed by haplotypes retrieved from vastly distanced populations, might be pointing the occurrence of a sole widely distributed species composed by scattered populations. Considering the fast-evolving character of mitochondrial genes, maintaining such a worldwide distribution with relatively low genetic distances between each population could be indicative of a constant spreading event associated to seabird hosts. As in *O. capensis*, genetic distances for the *O. spheniscus* clade showed a similar percentage of divergence (2.6%); however this species has a comparatively less expanded distribution, occurring to date only along the Chilean and Peruvian shores (Hoogstraal et al. 1985, González-acuña et al. 2008). Although the sequencing of more haplotypes is necessary to make an accurate conclusion, the genetic divergence exhibited by *O. spheniscus* suggests that gene flow could be slower or limited between different populations, at least for the currently sampled locations. Conversely, other two species analyzed in this study, *O. amblus* and *Ornithodoros* cf. *O. yunkeri*, have a less polymorphic set of haplotypes, which is reflected in low intraspecific genetic divergence (0.6% for both species) for the specimens sampled across a coastal line of ca. 2000 km in Chile and Peru. A constant translocation via seabird might be a possible cause to this fact.

6.4.4.3. *Coxiella*-like endosymbionts and tick phylogenies

One of the objectives of this study was to support that morphologic diagnoses of species from all populations were correctly stated by exploring one more line of genetic evidence: their related *Coxiella*-like specific endosymbionts. Although each of the four tick species was confirmed to represent a monophyletic group composed by several lineages, all with a specific *Coxiella*-like clone, independent phylogenetic reconstructions with the submitted sets of genes suggested differences in evolutionary histories between both groups of organisms. The phylogenetic evidence exposed in this study, corresponds to a tip of the whole underlying evolutionary history involving ticks and *Coxiella*-like endosymbionts, so the addition of new terminals to both combined phylogenies could bring an insightful view on the reasons explaining not completely congruent evolutionary histories. Anyway, by genetic analyses in different *Ornithodoros* spp., historical events of horizontal transmission between *Coxiella*-like organisms and their associated ticks have been suggested to occur, since closely related *Coxiella*-like endosymbionts can eventually occur in distantly related tick species (Duron et al. 2015, 2017).

6.5. CONCLUSIONS

In this study, a large sampling effort was conducted in order to identify seabird soft ticks along the coasts of Chile and Peru, and some insular territories from Brazil. The results were clear, and pointed the occurrence of at least four species in different islands and shore ecosystems along the prospected localities. By inserting the genetic identities of the species diagnosed in this study into a phylogenetic context including the vast majority of available Argasidae sequences, *O. capensis* s. l. forms a paraphyletic group. To confirm this trend, the complete array of species that constitutes the group must be subjected to a phylogenetic analysis. Unfortunately, five species of the group, namely *O. cheikhii*, *O. coniceps*, *O. collocallie*, *O. muesebecki*, and *O. yunkerii*, still lack any genetic sequences in order to perform such an analysis.

The comparison of larval stages of *O. amblus*, *O. spheniscus* and *Ornithodoros* cf. *O. yunkerii*, confirmed that they share a highly similar phenotype. Considering this, a morphological diagnosis using adult stages, with special

emphasis in the topology and macro-anatomy of dorsal disks is informative to discriminate between different species.

Although in this study ticks and their associated *Coxiella*-like endosymbionts did not exhibit completely congruent phylogenetic reconstructions, specific clones of *Coxiella* grouped exclusively with each one of the analyzed species of soft ticks. Therefore, even though both organisms might not share a single evolutionary history, the characterization of *Coxiella*-like genes still constitutes an additional tool of taxonomic importance in order to define genetic boundaries between closely related species of ticks.

6.6. REFERENCES

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7. CONCLUSIONS

When this study started back in 2014, seven species in the Argasidae and 12 in the Ixodidae family composed Chilean diversity of ticks. Although no additional species were included for the Ixodidae, the results herein presented add at least five new taxa to the Argasidae, since three new species, *Ornithodoros atacamensis*, *Ornithodoros* n. sp. 2, and *Ornithodoros* n. sp. 1 were described, and *Argas persicus* and *Ornithodoros capensis* expanded their distribution to Chilean territory. Moreover, an *Argas* sp. morphologically closely related to *Argas cucumerinus* was collected in the north of the country, and two putative new species, *Ornithodoros* cf. *O. rioplatensis* and *Ornithodoros* cf. *O. yunkeri* are still pending for an appropriate description, that will specially need the performance of cross-mating experiments with morphologically and genetically related species to shed light on their taxonomic classification.

By the molecular analyses, the results of this study allow to state that native Chilean ticks do harbor microorganisms of *Borrelia*, *Coxiella*, *Rickettsia* and *Hepatozoon* genus. Although through conventional PCR and subsequent sequencing, DNA of *Anaplasma*, *Ehrlichia* and “*Candidatus* Neoehrlichia” was also detected, the results are still partial, and need further study, mainly in the obtention of longer sequences for the targeted 16S rRNA genes. Otherwise, a generic identity could not be reliably assigned. The assessment of phylogenetic relatedness between the detected agents and tick microorganisms of known pathogenic roles, was conclusive for some species, and currently it can be stated that: 1) borreliae of the Recurrent Fever Group and Lyme Disease group are harbored by *Ornithodoros spheniscus* and *Ixodes* spp. (i. e. *Ixodes* cf. *I. sigelos*, and *Ixodes* cf. *I. auritulus*), respectively; 2) rickettsiae of the Spotted Fever Group (SFG), namely *Rickettsia amblyommatis*, infects *O. atacamensis*, and two new rickettsial agents phylogenetically close related with each other and basally positioned within the SFG, infect *Ornithodoros microlophi* and *Ornithodoros* cf. *O. rioplatensis*. On the other hand, *Hepatozoon* spp. found in *Ixodes* cf. *I. sigelos* and *O. atacamensis* are unrelated with any disease causing-*Hepatozoon* spp. such as *Hepatozoon canis*, and *Hepatozoon felis*, yet pathogenic roles in their vertebrate hosts should not be discarded.

Finally, sequences of *Coxiella*-like endosymbionts genes characterized from seabird soft ticks belonging to the *O. capensis* s. l. do constitute useful taxonomic tools in order to delimit different species of this group in South America. This fact was supported even for vastly distanced populations of ticks, since monophyletic groups of *Coxiella* clones mirrored monophyletic groups of associated ticks in four specific situations.