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Chlamydiaceae-Like Bacterium in Wild Magellanic Penguins (*Spheniscus magellanicus*)

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Abstract: The family *Chlamydiaceae* is comprised of obligate intracellular bacteria, some of which are significant pathogens of humans and domestic animals. Magellanic penguins (*Spheniscus magellanicus*) are susceptible to *Chlamydia psittaci* outbreaks in captivity, and serological surveys detected antibodies against *C. psittaci* (or antigenically similar organisms) in wild populations of this species. To investigate the occurrence of *Chlamydiaceae* in wild Magellanic penguins, 167 cloacal swabs were collected from apparently healthy individuals at four breeding colonies along the coast of Chubut, Argentina. Real-time PCR revealed the presence of DNA from *Chlamydiaceae*-like bacteria in 61 cloacal swabs (36.5%). Detection did not correlate to nutritional status or to meaningful hematological abnormalities. Isolation in monkey kidney cells was unsuccessful, but DNA sequences for the 16S–23S rRNA region were obtained from one sample. Phylogenetic analysis showed a close relationship to unidentified *Chlamydiaceae*-like bacteria found in chinstrap penguins (*Pygoscelis antarcticus*) in Antarctica and seagulls in France and the Bering Sea, and to *Chlamydiifrater* spp. isolated from flamingos in France. Further studies are necessary to clarify the taxonomy and investigate the epidemiology, pathogenicity, and zoonotic potential of this group of *Chlamydiaceae*-like bacteria.

Keywords: chlamydiosis; microbiota; real-time PCR; seabird; South America; wild bird

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1. Introduction

The *Chlamydiaceae* family is comprised of Gram-negative obligate intracellular bacteria. Current taxonomy recognizes a single genus, *Chlamydia*, with 10 accepted species (*C. abortus*, *C. avium*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. suis*, and *C. trachomatis*) and several proposed or candidate species that have yet to be fully recognized under the International Code of Nomenclature of Prokaryotes (*C. buteonis*, *C. caviae*, *C. ibidis*, *C. isopodii*, *C. serpentis*, *Ca.* C. coralli, *Ca.* C. sanziniae, *Ca.* C. testudini) [1]. Additionally, three genera have been proposed for inclusion in the *Chlamydiaceae* family: *Ca.* Clavichlamydia (one candidate species: *Ca.* C. salmonicola) [2], *Ca.* Amphibiichlamydia

(two candidate species: *Ca*. A. ranarum and *Ca*. A. salamandrae) [3], and *Chlamydiifrater* (two proposed species: *C. phoenicopteri* and *C. volucris*) [4].

Chlamydia spp. are significant pathogens of humans and domestic animals, causing a variety of chronic or acute clinical presentations [5,6]. *C. psittaci*, the causative agent of psittacosis, stands out as the most prominent zoonotic pathogen in this group, having caused large epidemics in the early 20th century and still causing hundreds of human deaths on an annual basis [7]. Wild birds are the natural reservoirs of *C. psittaci*, and the bacterium has been isolated from more than 460 avian species with a global pooled prevalence of *c.* 20% [8,9]. Infected birds shed the bacterium in their feces, regurgitates, and nasal discharges, and infection may be transmitted through contaminated food or water, fecal dust inhalation, or consumption of infected carcasses [7,10]. Hawks seem to play a special role as incubator hosts that promote the transformation and homologous recombination between different chlamydial species, contributing to the emergence of novel pathogenic lineages [11]. Although human infection by *C. psittaci* is often linked to psittacine birds (parrots and parakeets), pigeons, and turkeys, other birds can also be a source of human exposure [7]. For example, human infection in the psittacosis epidemic of 1930–1938 in the Faroe Islands occurred through the capture, handling, and cooking of seabirds—specifically, northern fulmars (*Fulmarus glacialis*) [7,12].

Penguins are susceptible to *C. psittaci* infection, as demonstrated by an outbreak of avian chlamydiosis in Magellanic penguins (*Spheniscus magellanicus*) at a North American zoo [13]. In that case, the *C. psittaci* strain was not isolated or genetically characterized, but species identity was confirmed through immunohistochemistry and PCR [13]. Notwithstanding, whether or not *C. psittaci* or other *Chlamydia* spp. naturally infect wild penguins remains an open question. Serological surveys (complement fixation test) show that wild populations of various penguin species may present antibodies against *C. psittaci* [14–20], but it is unclear whether these results reflect exposure to *C. psittaci* or to other antigenically similar organisms [20,21]. Cameron [22] reported on the isolation of "a member of the Psittacosis-Lymphogranuloma Venereum group of viruses" from the lungs of an emperor penguin chick (*Aptenodytes forsteri*) found dead in Antarctica, but it is unknown whether this organism was *Chlamydia* sp. or another *Chlamydiales* or *Chlamydiales*-like organism. Recent attempts to detect DNA from *C. psittaci* in fecal samples from wild penguins with real-time PCR tests failed [18,23], but Isaksson and colleagues [21] succeeded in detecting DNA from *Chlamydiales* sp. in cloacal swabs from 7 of 105 chinstrap penguins (*Pygoscelis antarcticus*) sampled in the Antarctic Peninsula. Sequencing of a short segment of the 16S rRNA gene revealed that the organism found in chinstrap penguins was not *C. psittaci*, but was closely related to other unidentified *Chlamydiaceae*-like bacteria detected in fecal samples from gulls and kittiwakes (Laridae) sampled in the northern hemisphere and in Chile [21].

It is important to continue exploring the diversity and occurrence of non-classified *Chlamydiaceae* in wild birds to expand our understanding of their ecological relevance. In this study, we report on the detection and molecular characterization of a *Chlamydiaceae*-like bacterium in cloacal swabs from Magellanic penguins sampled at breeding colonies along the coast of Chubut province, Argentina.

2. Materials and Methods

2.1. Sample Collection

A total of 167 Magellanic penguins were sampled at four breeding colonies along the coast of Chubut province, Argentina (Table S1). Between 21 and 25 January 2014 (post-guard chick rearing stage of the species' annual cycle [24], penguins were sampled at Punta Norte/San Lorenzo (3 chicks, 13 adults; 42°04′31″ S 63°47′19″ W), Punta Tombo (22 adults; 44°03′08″ S 65°13′20″ W), Cabo Dos Bahías (20 adults; 44°53′59″ S 65°34′43″ W), and Isla Vernacci Fondo (16 adults; 45°09′36″ S 66°34′35″ W). Further sampling was conducted at Cabo Dos Bahías from 7–8 November 2015 (incubation stage, 28 adults) and from 19 October to 8 November 2016 (incubation stage, 26 adults), and at Punta Norte/San

Lorenzo on 15 December 2017 (guard chick rearing stage; 11 chicks, 28 adults). Penguins were not molting, appeared to be healthy, and were either resting in the colony or sitting on nests with chicks. Captures were not conducted at the edges of the colony, nor in areas within 200 m of tourist boardwalks or trails (further details provided by Gallo and colleagues [25]).

Penguins were caught, manually restrained, and cloacal swabs (polyester) were collected. Swabs were placed either in dry tubes (molecular analysis) or in a tube containing sucrose–phosphate–glutamate medium (molecular analysis and culture) [26]. Samples were placed in liquid nitrogen in the field and then transferred to −80 °C freezer until laboratory analysis at the Departmento de Bioquímica Clínica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. During the 2013/2014 breeding season, we also took body measurements (bill depth, bill length, and body mass) and collected blood samples (<1% of body mass, collected through jugular venipuncture) from adult penguins. A body mass–size residual (BMSR) was calculated for these birds based on body mass and bill length [27]. Blood samples were transferred to heparinized tubes and kept cool with ice packs, then processed within 4–6 h to quantify the packed cell volume, total plasma solids, and differential leukocyte counts (laboratory methods detailed by Gallo and colleagues [25]).

2.2. Culture, DNA Extraction, qPCR and Sequencing

Culture in monkey kidney cells (LLC-MK2) was attempted for the subset of samples stored in culture medium, following the procedures described by Sachse and colleagues [28]. For molecular analysis, swabs were placed in 1 mL sucrose–phosphate–glutamate medium, and 200 µL of this solution were subjected to DNA extraction using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Alameda, CA, USA) according to the manufacturer's instructions. Extracted DNA was stored at 4 °C. DNA extracted from *Chlamydia trachomatis* strain L2/434Bu was used as a positive control. Each sample was tested initially with a real-time polymerase chain reaction (qPCR) targeting the 23S rRNA gene of *Chlamydiaceae* using the methods described by Sachse and colleagues [29]. A subset (n = 41) of samples that were positive for this initial screening were then tested with a qPCR protocol targeting the outer membrane protein A (*ompA*) gene of C. *psittaci* using the methods described by Sachse and colleagues [29]. A subset (n = 9) of the samples that were positive for *Chlamydiaceae* sp. but negative for C. *psittaci* were further analyzed by Sanger sequencing, including a short signature segment of the 16S rRNA gene (278 bp, obtained with primers 16SIGF and 16SIGR2) and a longer segment comprising part of the 16S rRNA gene, the 16S/23S intergenic spacer and part of the 23S rRNA gene (933 bp, obtained with primers 16SF2 and 23SIGR) using the methods described by Sachse and colleagues [28]. Primers and probes utilized are provided in Table S2.

DNA sequences obtained in this study were deposited in GenBank (accession codes OL870320 and OL873322) and compared to publicly available sequences of the 16S–23S genome region of *Chlamydiales* spp. (Table S3). Sequences were aligned using the ClustalW algorithm [30] as implemented in MEGA 7.0.26 [31]. Phylogenetic relationships were evaluated through a Bayesian phylogenetic tree produced using MrBayes 3.2.7 [32]. The analyses considered the 16S–23S rRNA region with three partitions: complete 16S rRNA gene (1557 nt), 16S/23S intergenic spacer (372 nt), and complete 23S rRNA gene (3067 nt). The GTR + G + I model of nucleotide evolution was used as recommended by jModelTest 2.1.10 [33]. Two Markov chains were run simultaneously for 5 million generations that were sampled every 1000 generations, and the first 1250 trees (25%) were discarded as a burn-in step. Pairwise evolutionary divergence (ED) was estimated with MEGA 7.0.26 using the maximum composite likelihood model with a gamma-distributed rate variation among sites (shape parameter = 1) and pairwise deletion of ambiguous positions.

2.3. Statistical Analysis

Data analysed in this study is provided in File S1. Chi-square tests were used to compare the frequency of positive *Chlamydiaceae* qPCR results among: (a) adult penguins sampled at four breeding colonies during the 2013/2014 season, and (b) three different years for adult penguins sampled at Cabo Dos Bahías. Fisher's exact tests were used to compare the frequency of positive *Chlamydiaceae* qPCR results among: (a) chicks and adults sampled at Punta Norte/San Lorenzo (pooled data from two breeding seasons), and (b) swabs stored in dry tubes or tubes containing culture medium (pooled data from samples collected at Cabo Dos Bahías in 2015/2016 and Punta Norte/San Lorenzo in 2017/2018). Physiological variables (body mass, BMSR, packed cell volume, plasma total solids, heterophil percentage, lymphocyte percentage, eosinophil percentage, basophil percentage, and heterophil-to-lymphocyte ratio) were tested for normality (Anderson–Darling test), and then compared between individuals with positive and negative results for the *Chlamydiaceae* qPCR (two-sample t-test when normally distributed, Mann–Whitney test when not normally distributed). Significance level was 0.05 for all tests.

3. Results

Sixty-one of 167 cloacal swabs (36.5%; 95% confidence interval = 29.5–44.0%) obtained from Magellanic penguins sampled at four breeding colonies along the coast of Chubut province, Argentina, were positive for a qPCR test targeting the 23S rRNA gene of *Chlamydiaceae*. Positive samples had an average quantification cycle (CQ) of 36.5 $\pm$ 3.0 (mean $\pm$ SD, range = 24.3–42.0; Table S1). *Chlamydiaceae* prevalence was not different among sampling years (χ^2 = 2.818, df = 2, p = 0.244), among breeding colonies (χ^2 = 6.081, df = 3, p = 0.108), or between chicks and adults (p = 0.361). There was no difference in the *Chlamydiaceae* prevalence between swabs stored in dry tubes compared to those stored in tubes containing the sucrose–phosphate–glutamate medium (p = 0.204). There were no significant differences in the hematological variables of individuals with positive and negative *Chlamydiaceae* qPCR results, with the exception of total plasma solids, which were marginally higher in positive individuals (53.8 $\pm$ 7.1 g/L compared to 50.0 $\pm$ 8.4 g/L in negative individuals; t = $-$2.02, df = 63, p = 0.048).

Forty-one of the samples that were positive for the *Chlamydiaceae* qPCR (CQ = 36.6 $\pm$ 3.3, range = 24.3–42.0) were tested with a qPCR targeting the outer membrane protein A (*ompA*) gene of C. *psittaci*, and all were negative. PCR amplification and Sanger sequencing of the 16S–23S rRNA region was attempted for a subset of nine samples collected at Cabo Dos Bahías in the 2014/2015 breeding season that were positive for the *Chlamydiaceae* qPCR (CQ = 35.3 $\pm$ 4.5, range = 24.3–38.3) and negative for the *C. psittaci* qPCR. Sequencing was successful for only one of these samples (sample SM1420), which was obtained from an adult male penguin with the lowest CQ in the *Chlamydiaceae* qPCR (CQ = 24.3). Phylogenetic analysis of the sequences obtained from this sample (Figure 1 and Table S4) revealed the presence of an organism that is closely related to *Chlamydiales* sp. strain 136621, which was detected in cloacal swabs from chinstrap penguins sampled in Antarctica, with an evolutionary distance (ED) of 1.48%. These strains clustered with three other *Chlamydiales* sp. strains detected in cloacal swabs from seabirds: C122 (ED = 2.95%), which were detected in feces from glaucous-winged gulls (*Larus glaucescens*) and glaucous gulls (*Larus hyperboreus*) sampled in the Bering Sea, in cloacal swabs from kelp gulls (*Larus dominicanus*) sampled in Chile, and 12-3998_AO053 (ED = 3.54%) and 12-1761_J072 (ED = 3.90%), both detected in cloacal swabs from European herring gulls (*Larus argentatus*) sampled at a rehabilitation facility in France (Figure 1). This cluster of unidentified strains was, in turn, grouped with *Chlamydiifrater volucris* strain 15-2067_O50 (ED = 6.13%) and *Chlamydiifrater phoenicopteri* strain 14-2711_R47 (ED = 6.55%), both isolated from cloacal swabs from greater flamingos (*Phoenicopterus roseus*) at a zoo in France (Figure 1). Isolation in monkey kidney cells (LLC-MK2) was not successful.

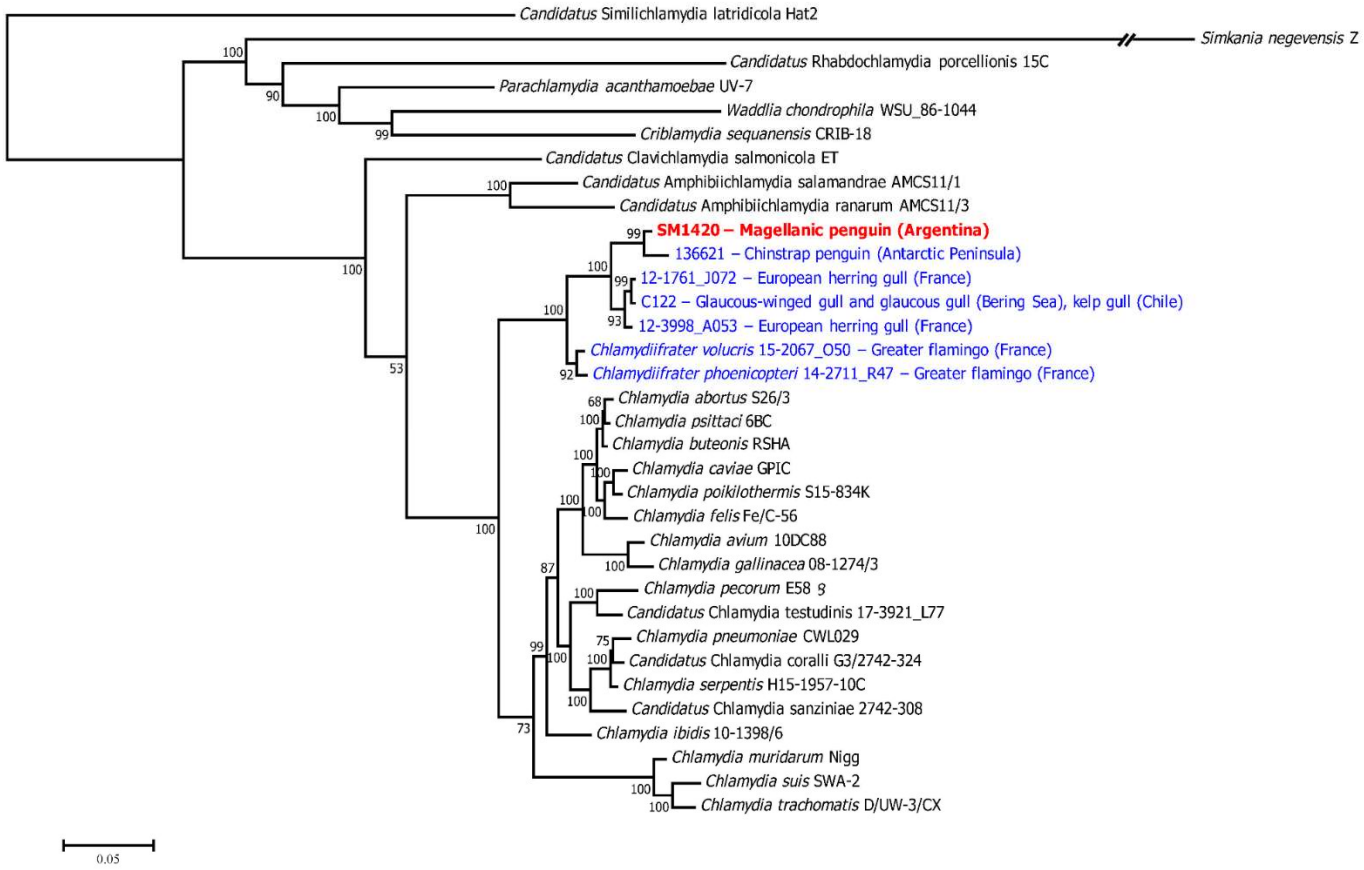

Figure 1. Bayesian phylogenetic hypothesis of the relationship between *Chlamydiales* spp. based on the 16S–23S rRNA region. Branch lengths are drawn proportionally to the extent of changes (scale bar is shown). Values adjacent to nodes represent posterior probabilities. The organism detected in this study is highlighted in red, and other *Chalmydiaceae*-like strains detected in aquatic birds are shown in blue.

4. Discussion

This is the first study to document the presence of *Chlamydiaceae*-like bacteria in wild Magellanic penguins. Phylogenetic analysis of the 16S–23S rRNA region revealed the organism detected in this study is closely related to the unidentified *Chlamydiales* strain found in cloacal swabs from chinstrap penguins sampled in Antarctica [21]. In turn, these strains from penguins are part of a sister clade of *Chlamydia* that comprises other unidentified *Chlamydiales* strains from gulls sampled in France, Chile, and the Bering Sea [21,34,35] and *Chlamydiifrater* spp. from captive flamingos in France [4]. As there is debate on the criteria to assign chlamydial species at the genus and family levels [4,36–38], it is unclear whether this group of organisms are part of the *Chlamydiaceae* family or if they represent a closely related, yet separate, family.

We observed a relatively high qPCR prevalence (36.5%) and relatively high CQ values (i.e., low DNA concentration). We also experienced a low success in gene sequencing and failed to isolate the bacterium in mammalian cell culture. Isaksson and colleagues [21] observed a similar pattern in chinstrap penguins at the Antarctic Peninsula. This suggests that *Chlamydiaceae*-like bacteria might be widespread in penguins, yet difficult to detect due to the low levels shed in feces. Considering most studies employing serological tests targeting antibodies against *C. psittaci* found relatively high seroprevalence in wild penguins [14–16,18–20], it is possible that these *Chlamydiaceae*-like bacteria elicit the production of antibodies that cross-react in vitro with *C. psittaci* antigens [39,40]. Further research is, therefore, warranted to evaluate the cross-reactivity of antibodies against *Chlamydia* and

Chlamydiaceae-like bacteria, and how this may interfere with the specificity of serological tests, especially in aquatic birds.

The Magellanic penguins sampled in this study were apparently healthy, and positivity to *Chlamydiaceae*-like bacteria did not correlate to nutritional status or to hematological variables, with the exception of total plasma solids that were marginally higher in positive individuals. Similarly, the chinstrap penguins studied by Isaksson and colleagues [21] also appeared healthy, as were the gulls and flamingos from which other closely related *Chlamydiaceae*-like strains were detected [4,34,35]. Isaksson and colleagues [21] suggested that the natural hosts of this group of *Chlamydiaceae*-like bacteria could be fishes, and that their detection in feces of aquatic birds would, thus, be an incidental finding related to their diet. Seeing that bacteria from this group have also been detected in flamingos [4], crustaceans also merit consideration as potential hosts. Ruling out this hypothesis of a non-avian host is relevant to understand the significance of these organisms to their apparent avian hosts. Nevertheless, current evidence suggests these bacteria are not significantly pathogenic to birds, at least not at the relatively low yields documented in these studies (as presumed from the generally high CQ values). However, pathogenicity in immunocompromised or otherwise ill birds should not be dismissed, as this has often been documented for *C. psittaci* and other *Chlamydia* spp. [41,42]. Likewise, apparently innocuous *Chlamydiaceae*-like bacteria could potentially become pathogenic when transferred to other hosts that are immunologically naïve, whether under natural (e.g., migration) or human-mediated circumstances (e.g., zoos or rehabilitation facilities). Further studies on the pathogenicity and health effects of these bacteria are, therefore, warranted. Additionally, considering that aquatic birds have a proven record as vectors of chlamydial infections to humans [7,12,41], it would also be expedient to investigate the zoonotic potential of these organisms.

Interestingly, Isaksson and colleagues [21] did not detect DNA from *Chlamydiales* in cloacal swabs from 159 Magellanic penguins sampled at Isla Magdalena, southern Chile. Coincidentally, Isla Martillo (*c.* 300 km southeast from Isla Magdalena) was the only colony of Magellanic penguins studied by Uhart and colleagues [20] where no antibodies against *Chlamydiales* were detected. These findings suggest that *Chlamydiaceae*-like bacteria could be absent/less prevalent in Magellanic penguin populations in the southernmost tip of South America (i.e., Tierra del Fuego region). Further studies should explore regional differences in the epidemiology of these bacteria, and what factors could be driving these patterns (e.g., climate, penguin diet, nesting density, nest microhabitat).

5. Conclusions

Our findings corroborate the existence of a group of *Chlamydiaceae*-like bacteria that can be detected in the feces and cloacal swabs of aquatic birds such as penguins, gulls, and flamingos. This group of bacteria is comprised of the recently proposed genus *Chlamydiifrater* as well as other strains that have yet to be formally described, including the strain detected in cloacal swabs from wild Magellanic penguins in this study. Further research is necessary to isolate these *Chlamydiaceae*-like bacteria and fully characterize them, and to investigate their epidemiology, pathogenicity, and zoonotic potential. The possibility that these organisms might interfere with serological tests used to study exposure to *Chlamydia* spp. in aquatic birds also merits investigation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d14090746/s1, Table S1: Diagnostic results for cloacal swabs of Magellanic penguins (Spheniscus magellanicus) tested using a real-time PCR test targeting the 23S rRNA gene of Chlamydiaceae. Table S2. Sequence of primers and probes employed. Table S3. GenBank accession codes of the DNA sequences evaluated in this study (16S rRNA gene, 16S/23S intergenic spacer, and 23 S rRNA gene). Table S4. Estimates of evolutionary divergence (number of base substitutions per site in the 16S–23S rRNA region) of Chlamydiaceae-like strains from aquatic birds.

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Institutional Review Board Statement: The study was conducted in accordance with all ethical guidelines for the use of wild birds in research as stipulated by the standards and policies of the Government of Argentina. Research procedures were revised and approved under permits by Dirección de Conservación y Áreas Protegidas and Dirección de Fauna y Flora Silvestre of Chubut Province, Argentina.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data analyzed in this study are provided in the supplementary materials. Sequence data are available in GenBank under accession numbers OL870320 and OL873322.

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Conflicts of Interest: The authors declare no competing interests.

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