diversity

MDPI

Article

Characterization of a *Pseudokeronopsis* Strain (Ciliophora, Urostylida) and Its Bacterial Endosymbiont "*Candidatus* Trichorickettsia" (*Alphaproteobacteria*, *Rickettsiales*)

Michele Castelli [1,*,†], Valentina Serra [2,†], Leandro Gammuto [2], Marcus V. X. Senra [3], Letizia Modeo [2,*] and Giulio Petroni [2,4,5]

1 Department of Biology and Biotechnology, University of Pavia, 27100 Pavia, Italy
2 Department of Biology, University of Pisa, 56126 Pisa, Italy
3 Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo Andre 09210-580, Brazil
4 CIME, Centro Interdipartimentale di Microscopia Elettronica, University of Pisa, 56100 Pisa, Italy
5 CISUP, Centro per l'Integrazione Della Strumentazione, University of Pisa, 56100 Pisa, Italy
* Correspondence: michele.castelli@unipv.it (M.C.); letizia.modeo@unipi.it (L.M.)
† These authors contributed equally to this work.

Abstract: Symbiotic associations between bacteria and ciliate protists are rather common. In particular, several cases were reported involving bacteria of the alphaproteobacterial lineage *Rickettsiales*, but the diversity, features, and interactions in these associations are still poorly understood. In this work, we characterized a novel ciliate protist strain originating from Brazil and its associated *Rickettsiales* endosymbiont by means of live and ultrastructural observations, as well as molecular phylogeny. Though with few morphological peculiarities, the ciliate was found to be phylogenetically affiliated with *Pseudokeronopsis erythrina*, a euryhaline species, which is consistent with its origin from a lagoon with significant spatial and seasonal salinity variations. The bacterial symbiont was assigned to "*Candidatus* Trichorickettsia mobilis subsp. hyperinfectiva", being the first documented case of a *Rickettsiales* associated with urostylid ciliates. It resided in the host cytoplasm and bore flagella, similarly to many, but not all, conspecifics in other host species. These findings highlight the ability of "*Candidatus* Trichorickettsia" to infect multiple distinct host species and underline the importance of further studies on this system, in particular on flagella and their regulation, from a functional and also an evolutionary perspective, considering the phylogenetic proximity with the well-studied and non-flagellated *Rickettsia*.

Keywords: *Rickettsiales*; rRNA-gene based phylogeny; flagella; symbiosis; intracellular bacteria; ciliate protists; taxonomy; Hypotrichia; ultrastructure; FISH

Citation: Castelli, M.; Serra, V.; Gammuto, L.; Senra, M.V.X.; Modeo, L.; Petroni, G. Characterization of a *Pseudokeronopsis* Strain (Ciliophora, Urostylida) and Its Bacterial Endosymbiont "*Candidatus* Trichorickettsia" (*Alphaproteobacteria*, *Rickettsiales*). *Diversity* **2022**, *14*, 1032. https://doi.org/10.3390/d14121032

Academic Editor: Genoveva F. Esteban

Received: 21 August 2022
Accepted: 23 November 2022
Published: 25 November 2022

1. Introduction

Protists and bacteria display a wide range of relationships, and may form complex microbial communities, with variable levels of interaction and integration [1,2]. Such associations may play important roles in the ecology and evolution of the involved bacteria [3], as well as on their hosts. Indeed, as underlined in a recent work in which bacterial endosymbionts were treated as taxonomic descriptors for a ciliate species of the genus *Euplotes*, symbionts can deeply influence their hosts in many aspects, even in their resulting morphology [4].

In general, protists harbour abundant and diverse bacterial endosymbionts (i.e., intracellular bacteria), including several representatives of lineages that also encompass human pathogens, such as *Rickettsiales*, *Legionellales*, and *Chlamydiae* [5–7]. Some protists are even able to host, at least temporarily, human pathogenic bacteria such as *Legionella* [8,9], thus potentially representing natural reservoirs. Those features have led several authors to infer a role of protists as melting pots for the evolution of potentially pathogenic bacteria, able to

invade multicellular eukaryotes, hence potentially acting as Trojan horses [10–12]. Several studies provided relevant contributions in this sense, investigating the mechanisms and dynamics of establishment of intracellular symbioses in protists such as ciliates [13–16].

In particular, multiple representatives of *Rickettsiales* are hosted by ciliate protists. Among those, the cases of "*Candidatus* (*Ca*.) Trichorickettsia mobilis" and "*Ca*. Gigarickettsia flagellata" are noteworthy. "*Ca*. Trichorickettsia" was found in association with multiple strains of several ciliate species (*Paramecium multimicronucleatum*, *P. nephridiatum*, *P. calkinsi*, *Euplotes aediculatus*) [17–19], while "*Ca*. Gigarickettsia" only in a single population of *Spirostomum minus* [17]. These two symbiont species are sister groups, and, in turn, together form the sister group of the lineage of *Rickettsia* and the recently described genus "*Ca*. Tisiphia" [20]. Genus *Rickettsia* in particular includes many pathogens vectored by hematophagous arthropods, causing various diseases in humans and other vertebrates, such as endemic typhus, epidemic typhus, and Rocky Mountain spotted fever [21].

Until recently, as still reported in the description in *Bergey's Manual of Systematics* of *Archaea* and *Bacteria* [22], all *Rickettsiales* were considered devoid of flagella. However, in the last decade, unexpectedly, several *Rickettsiales* were found to harbour flagella [23], including "*Ca*. Trichorickettsia" and "*Ca*. Gigarickettsia" [17,19], or flagellar genes [20]. Flagella likely date back to the *Rickettsiales* ancestor [24,25], but their role in strictly obligate intracellular symbionts, such as the vast majority of characterised *Rickettsiales* (though with at least one significant exception [26]) or even *Chlamydiae* [6], still needs to be elucidated. Multiple non-mutually exclusive functions have been hypothesised, either as actual motile organelles [5,24,27], possibly during horizontal transmission, or as mediators of host-symbiont interactions [23,24,28]

Among "flagellated" *Rickettsiales*, the case of "*Ca*. Trichorickettsia" and "*Ca*. Gigarickettsia" is peculiar, as, to our best knowledge, they are the only *Rickettsiales* bacteria for which flagellar-driven motility was actually observed [17]. Moreover, "*Ca*. Trichorickettsia mobilis" presents intraspecific variability for the presence of flagella, which resulted up to now quite consistent with genetic diversity [17,19].

In this work, we report the characterisation of a novel "*Ca*. Trichorickettsia" representative, discovered as endosymbiont of the hypotrich ciliate *Pseudokeronopsis* (Spirotrichea, Urostylida). This finding provides further information for understanding the intraspecific phenotypic variability of "*Ca*. Trickorickettsia" regarding host species specificity and flagellar repertoire. Additionally, the ciliate host itself is noteworthy and, according to morphological and molecular analyses, it likely represents a novel record of an euryhaline [29] *Pseudokeronopsis* species, i.e., *Pseudokeronopsis erythrina*, though with few distinctive morphological features, and is also the first member of urostylid ciliates recorded so far for hosting a *Rickettsiales* bacterium.

2. Materials and Methods

Pseudokeronopsis sp. strain PSqRJ01 was isolated from a water sample collected in Jacarepiá lagoon, located in Saquarema city, 107 km east from Rio de Janeiro, Brazil (22°54′53″ S, 42°25′43″ W). The strain was maintained in the laboratory inside an incubator at 19 ± 1 °C, with a 12 h light and a 12 h dark. It was fed as previously described [30] on the diatom *Phaeodactylum tricornutum*, in turn propagated as a monoclonal culture at 5‰ salinity.

Cells of *Pseudokeronopsis* sp. PSqRJ01 were examined in vivo for their behaviour and their morphological features under a stereomicroscope (WILD HEERBRUGG, Switzerland) and a Leitz Orthoplan (Weitzlar, Germany) Differential Interference Contrast (DIC) microscope equipped with a digital camera (Canon PowerShot S45), at a magnification of 10–50× and 100–1250×, respectively.

Approximately 100 cells were fixed in 70% ethanol, and DNA extraction was performed employing the NucleoSpinTM Plant II kit (Macherey-Nagel, Düren, Germany).

The 18S rRNA gene of *Pseudokeronopsis* sp. PSqRJ01 was amplified and sequenced with universal eukaryotic primers (18S F9 Euk—[31]—18S R1513 Hypo—[32]), as previously

described [19]. The internal transcribed spacer (ITS) was amplified with primers 18S F919 [33] and RGD2 [34] as previously described [19].

Based on preliminary fluorescence microscopy observations, the 16S rRNA gene of the bacterial symbiont of *Pseudokeronopsis* sp. PSqRJ01 was amplified with primers RickFla_F69 and Rick_R1455, as previously described [17], and sequenced with internal primers [35].

Pseudokeronopsis sp. PSqRJ01 cells were washed in sterile water prior to fixation in 4% (*v*/*v*) formaldehyde (in PBS 1×) on microscope slides. The protocol by Manz and co-authors [36] was followed for the subsequent hybridisation, using multiple combinations of probes with different specificities, in particular the probe EUB338 [37] (5′-GCTGCCTCCCGTAGGAGT-3′), targeting over 90% of domain *Bacteria*, the ALF1b (5′-CGTTCGYTCTGAGCCAG-3′) [36], targeting *Alphaproteobacteria*, and the "*Candidatus* Trichorickettsia"-specific probe TrichoRick_142 (5′-GTTTCCAAATGTTATTCCATAC-3′) [17]. Fixed cells were simultaneously investigated under UV-light after staining with DAPI dye.

Pseudokeronopsis sp. PSqRJ01 cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 45 min, briefly rinsed in the same buffer, and post-fixed in 1.5% aqueous osmium tetroxide in distilled water for 45 min at room temperature. Then, cells were dehydrated and processed as elsewhere described [38]. Briefly, cells were embedded in an Epon-araldite mixture and the obtained blocks sectioned with an RMC PowerTome X ultra-microtome; sections were placed on copper grids and stained with uranyl acetate and lead citrate. Samples were observed using a JEM-100SX-TEM and a JEM-F200 HR FEG-TEM (both JEOL Ltd., Tokyo, Japan).

The 18S rRNA gene sequence of *Pseudokeronopsis* sp. PSqRJ01 was aligned with the automatic aligner of the ARB software package version 5.5 [39] on the SSU ref NR99 SILVA database [40], manually updated with latest released sequences.

For the phylogenetic analysis, 32 18S rDNA sequences belonging to representatives of the genus *Pseudokeronopsis* (or closely related lineages) were selected for the ingroup, and nine sequences belonging to other urostylids were selected for the outgroup, for a total of 41 sequences.

After manual editing to optimise base pairing in the predicted rRNA stem regions, the alignment was trimmed at both ends to the length of the shortest sequence. The resulting matrix contained 1675 nucleotides and was used for phylogenetic reconstructions.

The optimal substitution model was selected with jModelTest 2.1 [41] according to the Akaike Information Criterion (AIC). The maximum likelihood (ML) tree was inferred with PHYML version 2.4 [42] from the ARB package, performing 100 pseudo-replicates. Bayesian inference (BI) tree was inferred with MrBayes 3.2 [43], using three runs, each with one cold and three heated Monte Carlo Markov chains, iterating for 1,000,000 generations with a burn-in of 25%.

For the phylogenetic analysis of the endosymbiont, the 16S rRNA gene of this bacterium and closely related sequences retrieved from NCBI nucleotide were aligned with the automatic aligner of the ARB software package to the SSU ref NR99 123 SILVA database, and the alignment was manually refined to optimise base pairing in the predicted rRNA structure. The final selection for the phylogeny included 32 total organisms, namely eight "*Ca*. Trichorickettsia mobilis", twenty other representatives of family *Rickettsiaceae*, and, as outgroup, four other members of the order *Rickettsiales*. The alignment of selected sequences was trimmed at both ends to the length of the shortest sequence and keeping all internal positions, resulting in 1360 nucleotide columns. The best substitution model was selected with jModeltest according to the AIC. ML and BI inference (BI) phylogenies were then estimated, respectively, with PHYML, with 1000 pseudo-replicates, and MrBayes, with three runs of three heated and one cold chain iterating for 1,000,000, applying a burn-in of 25%. Identity values within "*Ca*. Trichorickettsia" and "*Ca*. Gigarickettsia" were calculated with the same full matrix as phylogeny, and with a modified matrix excluding their typical gene insert [17,19] (1095 total nucleotide sites).

3. Results

3.1. Characterization and Identification of the Host

Pseudokeronopsis sp. PSqRJ01 cells (Figure 1) were 140–240 × 17–37 µm in vivo (mean value: 203 × 23 µm, measured on more than 20 undeformed individuals). A buccal field occupied one third to one fourth of the cell length (Figure 1A). Cells appeared dorsoventrally flattened, elliptical in shape and elongated, with rounded ends, the posterior one sometimes larger than the anterior one; cell body was quite flexible (Figure 1A). A single contractile vacuole was present in the posterior half of the cell (Figure 1A). Numerous spherical pigment granules (coloured in red blood to brick red to brownish; diameter: ~1.7 µm) were present in the cortex (Figure 1). They were especially, but not exclusively, distributed around the different cell ciliature components (i.e., each ciliary unit of adoral zone membranelles, cirri, and dorsal kineties) (Figure 1B–D). Additionally, numerous ellipsoidal colourless granules (~1.5–1.8 × 0.9 µm), were observed densely distributed beneath the cortex and in the cytoplasm (Figure 1C,D). Cells spent most of the time crawling on the substrate

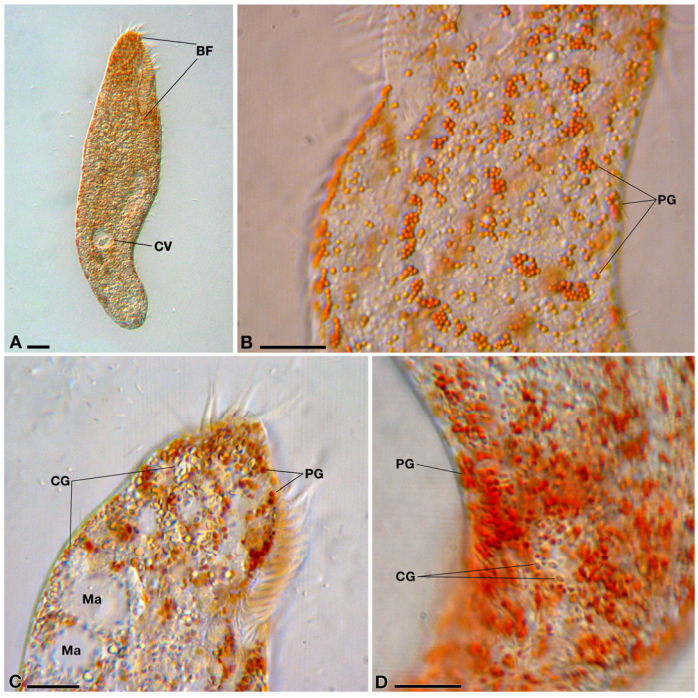

Figure 1. DIC images of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 in vivo. (**A**) A whole cell. The buccal field (BF) occupies between a third and a fourth of the cell length. A single contractile vacuole (CV) is present in the posterior half of the cell; (**B**) a lateral view just below the BF level, between the ventral (on the left) and the dorsal (on the right) cell side: pigment granules (PG) are generally associated with each component (i.e., ciliary unit) of the cell ciliature; (**C**) the anterior cell region. Both PG and ellipsoidal colourless granules (CG) are present. Two macronuclear beads (Ma) are also visible; (**D**) many PG are also distributed randomly throughout the cell cortical region. Bars stand for 10 µm.

Transmission electron microscopy showed additional features (Figure 2), such as: 1. the structure of the macronuclei (size: ~4 × 2 µm), which showed irregular chromatin bodies and large nucleoli (diameter: ~0.75 µm) (Figure 2A); 2. ovoidal mitochondria (length: ~1–1.5 µm), (Figure 2A,B), sometimes associated with ribosomes (Figure 2B) (likely belonging to the rough endoplasmic reticulum cisternae); 3. medium to large lipid droplets (diameter: ~1.0–2.0 µm) and numerous bodies of polysaccharide reserve (Figure 2A–C); 4. several sparse, endosymbiotic bacteria exhibiting a common single morphotype (see below for details), encircled by a white halo and embedded in the host's cytoplasm (i.e., not enclosed in any symbiosomal vacuole) (Figure 2).

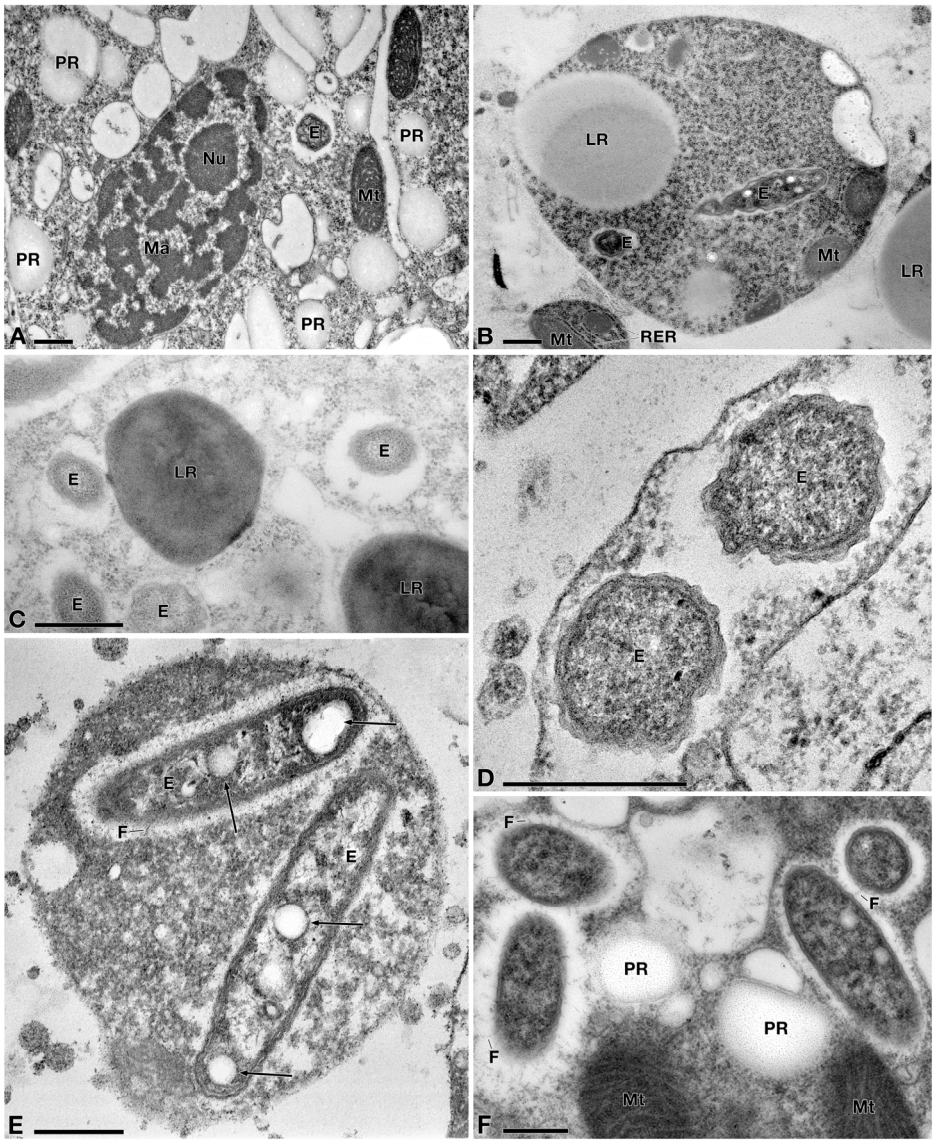

Figure 2. TEM images of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 and its bacterial endosymbionts "*Ca*. Trichorickettsia mobilis". (**A**) In the cytoplasm, a macronucleus (Ma) with a large nucleolus (Nu), several ovoidal mitochondria (Mt) and polysaccharide reserves (PR), and endosymbionts (E) are visible; (**B**) Mt sometimes are associated with ribosomes likely belonging to the rough endoplasmic reticulum (RER) cisternae; (**C**) lipid reserves (LR) often correspond to medium to large-sized droplets. Three endosymbiotic bacteria in the neighbourhood of two LR. They are encircled by a white halo and embedded in the host's cytoplasm; (**D**) two cross-sectioned endosymbiotic bacteria showing their two-membrane cell wall; (**E**) bacteria are rod shaped, and include areas with variable electron density, electron-lucid granules (arrows), and filaments (DNA); (**F**) sometimes bacteria show flagella (F) distributed all around the cell. Bars stand for 0.5 μm.

The combined partial 18S rRNA gene, ITS and partial 28S rRNA gene of *Pseudokeronopsis* sp. PSqRJ01 were 2842 bp in length. The highest identity (~99.8%, three mismatches and no gaps over 1732 aligned positions) was found with an 18S rRNA gene sequence labelled as *Pseudokeronopsis erythrina* strain TL-1 (KX459375.1), while, considering the full available 18S-ITS-28S sequence, the best hit was *Pseudokeronopsis rubra* INHC29 (HQ228548.1 overall identity 97.7% with 59 mismatches and 6 gaps, while the corresponding identity in the 18S was 99.5%, with 8 mismatches and no gaps).

Phylogenetic analyses showed coherent topologies between ML and BI trees (Figure 3). The relationships among the *Pseudokeronopsis* species were not fully resolved by the present analysis, considering the relatively poor support for some nodes. Nevertheless, *Pseudokeronopsis* sp. PSqRJ01 resulted within the monophyletic *P. erythrina* clade with high support (92 ML | 0.99 BI), and closely related to *P. rubra* and *P. flava*.

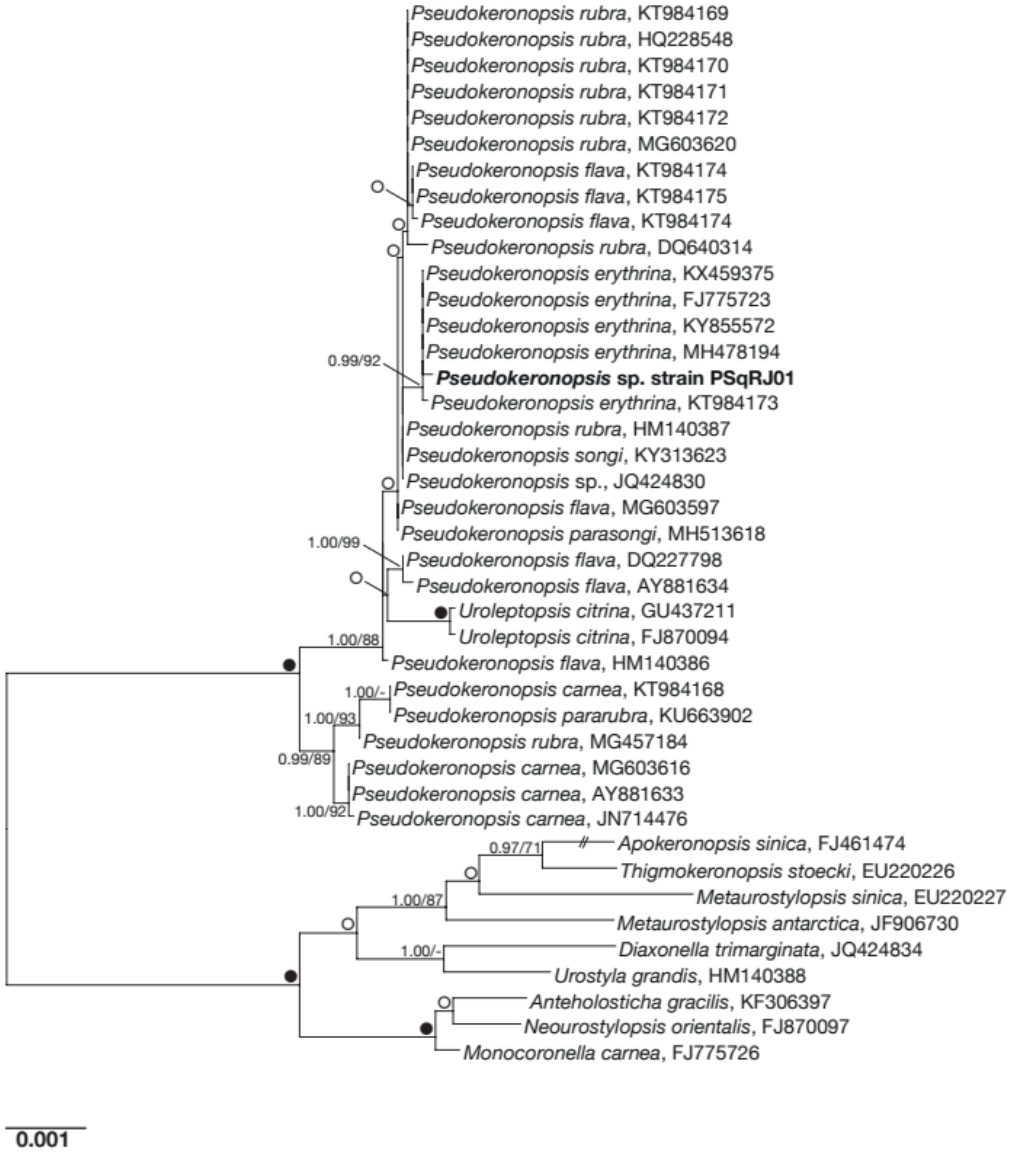

Figure 3. Maximum likelihood tree of the genus *Pseudokeronopsis* based on 18S rDNA sequences. The phylogenetic position of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 is shown. Numbers associated with nodes represent posterior probability from Bayesian inference (BI) and bootstrap value from maximum likelihood (ML) analyses, respectively (only values of BI $\geq$ 0.80 and ML $\geq$ 70% are shown). Black dots represent the highest statistical support (BI = 1.00 and ML = 100); white dots indicate non-significant statistical support (BI < 0.80 and ML < 70%). The sequence obtained in the present work is in bold.

To sum up, the results of the morphological investigation on both in vivo and TEM processed specimens, showing that most of our specimen's features matched with those of *P. erythrina* as originally described by [44] (see Discussion below), and the findings of the molecular analyses support each other. Thus, from now on we will refer to PSqRJ01 strain as *Pseudokeronopsis* cfr. *erythrina* PSqRJ01.

3.2. Characterization of the Bacterial Symbiont

Fluorescence in situ hybridisation experiments revealed the presence of several cells of an alphaproteobacterial endosymbiont in the cytoplasm of *Pseudokeronopsis* cfr. erythrina PSqRJ01 (Supplementary Figure S1).

The obtained *bacterial* 16S rRNA gene sequence was 1527 bp long with a 100% identity with "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva" endosymbiont of *P. calkinsi* strain CyL 8-33 (MF039744.1). Phylogenetic analyses fully confirmed the assignment of the PSqRJ01's symbiont to "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" (1.00 BI | 99 ML. Figure 4). Moreover, phyletic relationships between "*Ca*. Trichorickettsia mobilis" subspecies, i.e., in particular "*Ca*. Trichorickettsia mobilis subsp. mobilis" and "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" as sister groups (0.96 BI/93 ML), as well as within *Rickettsiaceae* in general, were consistent with other 16S rRNA gene-based and phylogenomic studies [19,45–49].

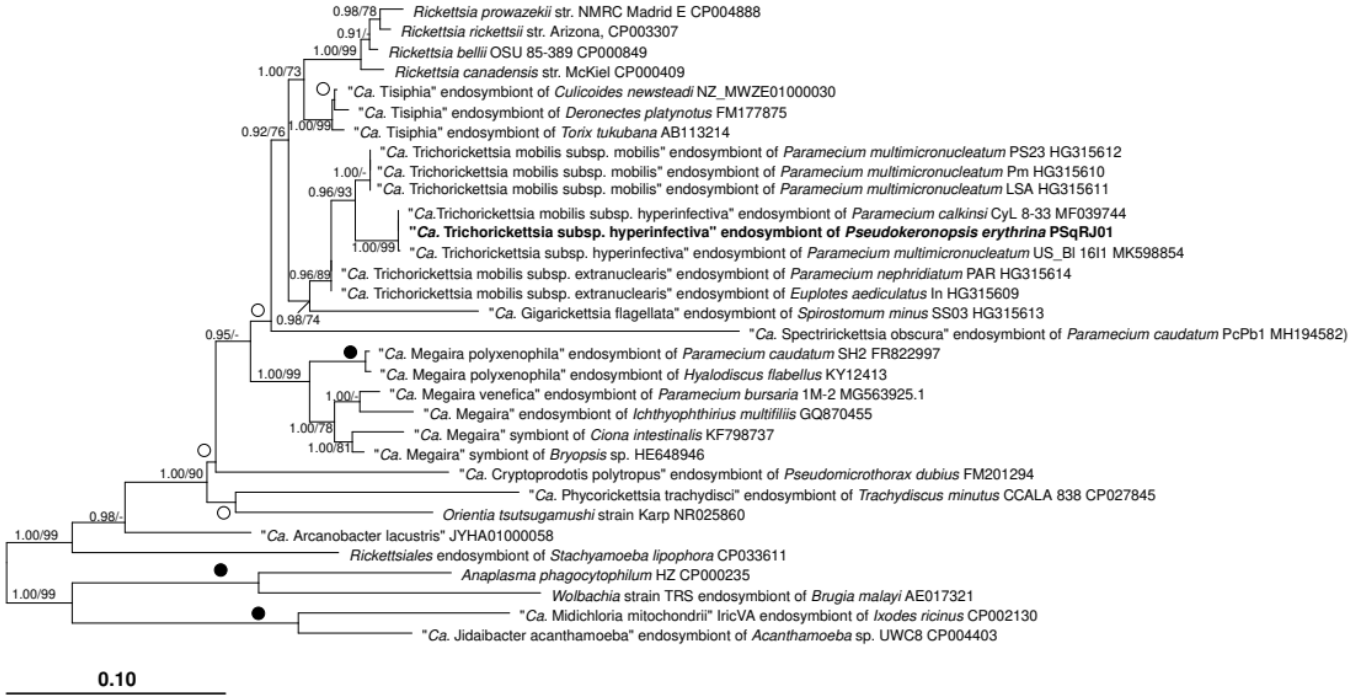

Figure 4. Maximum likelihood tree of the family *Rickettsiaceae* based on 16S rDNA sequences. The phylogenetic position of the bacterial symbiont of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 is shown. Numbers associated with nodes represent posterior probability from BI and bootstrap value from ML analyses, respectively (only values of BI $\geq$ 0.80 and ML $\geq$ 70% are shown). Black dots represent the highest statistical support (BI = 1.00 and ML = 100); white dots indicate non-significant statistical support (BI < 0.80 and ML < 70%). The sequence obtained in the present work is in bold.

In terms of sequence identity, the 16S rRNA genes of "*Ca*. Trichorickettsia mobilis" were highly homogeneous within each subspecies, with 100% identities both in the "full sequence" and the "no insert matrix" (Tables 1 and 2). On the other side, most of the observed divergence between subspecies is concentrated in the insert regions (Table 1), which is putatively non-functional, as identities not considering the insert are all rather high (≥99.5%) (Table 2), consistent with previous studies [17,19], and with the species threshold of 98.65–98.7% [50].

Table 1. Percent identity values of the 16S rRNA genes of "*Ca*. Trichorickettsia" on the "full-sequence" matrix (1360 nucleotide positions). The newly characterized sequence of the endosymbiont of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 is highlighted in bold. Full identity values are highlighted in bold.

Organism	1.	2.	3.	4.	5.	6.	7.	8.
1. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* PS23 HG315612	**100**	**100**	**100**	97.7	97.7	97.6	98.4	98.4
2. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* Pm HG315610		**100**	**100**	97.7	97.7	97.6	98.4	98.4
3. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* LSA HG315611			**100**	97.7	97.7	97.6	98.4	98.4
4. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Paramecium calkinsi* CyL 8-33 MF039744				**100**	**100**	**100**	97.3	97.3
5. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Pseudokeronopsis erythrina* PSqRJ01					**100**	**100**	97.3	97.3
6. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Paramecium multimicronucleatum* US_Bl 16I1 MK598854						**100**	97.2	97.2
7. "*Ca*. Trichorickettsia mobilis subsp. extranuclearis"—*Paramecium nephridiatum* PAR13 HG315614							**100**	**100**
8. "*Ca*. Trichorickettsia mobilis subsp. extranuclearis"—*Euplotes aediculatus* In HG315609								**100**

Table 2. Percent identity values of the 16S rRNA genes of "*Ca*. Trichorickettsia" on the "no insert" matrix (i.e., excluding the typical insert present in these bacterial endosymbionts: 1095 nucleotide positions). The newly characterized sequence of the endosymbiont of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 is highlighted in bold. Full identity values are highlighted in bold.

Organism	1.	2.	3.	4.	5.	6.	7.	8.
1. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* PS23 HG315612	**100**	**100**	**100**	99.5	99.5	99.5	99.7	99.7
2. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* Pm HG315610		**100**	**100**	99.5	99.5	99.5	99.7	99.7
3. "*Ca*. Trichorickettsia mobilis subsp. mobilis"—*Paramecium multimicronucleatum* LSA HG315611			**100**	99.5	99.5	99.5	99.7	99.7
4. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Paramecium calkinsi* CyL 8-33 MF039744				**100**	**100**	**100**	99.5	99.5
5. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Pseudokeronopsis erythrina* PSqRJ01					**100**	**100**	99.5	99.5
6. "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva"—*Paramecium multimicronucleatum* US_Bl 16I1 MK598854						**100**	99.5	99.5
7. "*Ca*. Trichorickettsia mobilis subsp. extranuclearis"—*Paramecium nephridiatum* PAR13 HG315614							**100**	**100**
8. "*Ca*. Trichorickettsia mobilis subsp. extranuclearis"—*Euplotes aediculatus* In HG315609								**100**

Unfortunately, after some initial experiments, *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 was lost and FISH experiments with more specific probes could no longer be performed. However, TEM investigations disclosed that 1, the endosymbionts exhibited a common morphology, uniform between them, including a two-membrane cell wall typical of Gram-negative bacteria (Figure 2) and a rod-shaped silhouette (size: ~2.0 × 0.4 µm) with generally rounded ends (Figure 2B,E,F); 2, bacterial cytoplasm was not homogeneous, i.e., it included areas with variable electron density, electron-lucid granules (one to three in number per bacterium) (diameter: ~0.5–0.7 µm), and filaments (DNA) (Figure 2B,E,F); 3, on several occasions thin flagella (diameter: ~12–15 nm; length ~60–90 nm) were visible (Figure 2E,F), often distributed all around the bacterial cell (Figure 2F). Thus, the appearance of the cytoplasmic bacterial endosymbionts of PSqRJ01 was consistent with previous reports of "*Ca*. Trichorickettsia mobilis" [17–19,38,51].

4. Discussion

In this work, we characterised by a multidisciplinary approach (in vivo and ultrastructural observations, molecular phylogeny) a strain of the ciliate protist *Pseudokeronopsis*, isolated from a sample coming from Brazil, and its associated *Rickettsiales* bacterial symbiont, affiliated to "*Ca*. Trichorickettsia". While reports of *Rickettsiales* (including "*Ca*. Trichorickettsia) in association with ciliates and other protists are rather common [5,26,45,47,49,52–55], to our best knowledge, this represents the first record of a host belonging to the genus *Pseudokeronopsis*, and, in general, to urostylid ciliates. It also represents the first record of a "*Ca*. Trichorickettsia" from South America. The characterisation of the host and of the symbiont will be discussed below separately, in a comparison with known relative organisms.

For what concerns the host taxonomy, it is well known that species attribution in the genus *Pseudokeronopsis* is difficult, due to a high interspecific morphological similarity [44,56–60]. Indeed, as evidenced in Figure 3, several sequences attributed to different species do not form monophyletic clades, suggesting a possible misidentification of few isolates (or the use of slightly different identification criteria by different researchers). Moreover, within the genus *Pseudokeronopsis* 18S rDNA sequences are poorly informative in resolving phylogenetic relationships, due to extremely low distances. Nevertheless, in the case of the herein characterised *Pseudokeronopsis* sp. PSqRJ01, 18S rRNA gene data clearly indicated a strongly supported affiliation to the *P. erythrina* clade. Unfortunately, due to an incomplete reference database, we could not use the obtained ITS and 28S rRNA gene sequences for species identification and for an improved phylogenetic resolution within the genus. Nevertheless, our data may offer such a possibility in future studies, being the ITS more and more used for species delimitation in ciliates, e.g., [61–65]. As for the morphological analyses, they were overall supportive for a relatedness to *P. erythrina* (Chen et al., 2011). Only the following two differences with the original description of *P. erythrina* [44] were noticed: 1, colourless granules were found to be densely distributed in the PSqRJ01 cytoplasm (likely not coincident with the blood cell-shaped granules found in other species of the genus; see [57,58]), while they are absent in *P. erythrina*; 2, a single contractile vacuole was observed, as opposed to, generally, two in *P. erythrina*. Taking all the above together and considering that a careful taxonomic revision of genus *Pseudokeronopsis* would be beyond the aims of the present study, we opted to conservatively refer to the novel strain as to *Pseudokeronopsis* cfr. *erythrina* PSqRJ01. *Pseudokeronopsis erythrina* is considered a euryhaline species [29], having been retrieved from both brackish and freshwater environments in China and Italy, respectively [29,44]. Interestingly, the finding of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 in the Saquarema lagoon, which shows significant spatial and seasonal variations in salinity [66], can be seen as consistent with previous observations on the autoecology of *P. erythrina*.

For what concerns the presence of intracellular bacterial symbionts in *Pseudokeronopsis* cfr. *erythrina* PSqRJ01, molecular analyses clearly indicate the presence of "*Ca*. Trichorickettsia mobilis". Specifically, the symbiont is affiliated with full support to "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" (1.00 BI|99 ML). Such finding expands the known host range for this bacterium. Indeed, "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" was up to now retrieved in natural association only with members of genus *Paramecium* [19,38], similarly to, in general, most representatives of the other subspecies of "*Ca*. Trichorickettsia mobilis" as well [17]. Therefore, it is implied that this bacterium displays the ability to shift horizontally between different host species in natural conditions, suggesting a certain flexibility in terms of host preference. This is consistent with general inferences on other *Rickettsiales* species, e.g., "*Ca*. Megaira polyxenophila" [20,47,52], and, interestingly, also with previous laboratory experiments on another "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva", naturally hosted by *Paramecium multimicronucleatum*, and able to invade, at least transiently, a metazoan host (the planarian *Dugesia japonica*) [38].On the other hand, interestingly, the association of "*Ca*. Trichorickettsia mobilis" with the ciliate *P. multimicronucleatum* was reported to be quite stable in laboratory conditions,

even in the case of antibiotic/antimicrobial treatments [18,51]. The unfortunate loss of the host strain *Pseudokeronopsis* cfr. *erythrina* PSqRJ01 did not allow us to perform further investigations that could rule out the presence of additional, phylogenetically distinct, intracellular bacteria. Nevertheless, combined together, multiple available evidence, in particular the preliminary FISH experiments with alphaproteobacterial probes (Supplementary Figure S1) and the uniform morphology and ultrastructure (Figure 3), consistent with multiple previous studies on "*Ca*. Trichorickettsia" [17–19,38,51], indicate that such possibility is unlikely.

Similarly to several other "*Ca*. Trichorickettsia" [17–19,38,51], the PSqRJ01 symbiont was often found to bear flagella (Figure 2). The presence/absence pattern of flagella within "*Ca*. Trichorickettsia mobilis" is peculiar, so far with all specimens of "*Ca*. Trichorickettsia mobilis subsp. mobilis" and "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" (including the herein characterised symbiont) being flagellated, while those of "*Ca*. Trichorickettsia mobilis subsp. extranuclearis" are non-flagellated [17–19,38,51]. There might be also a potential variability in flagellar abundance (both in terms of proportion of flagellated cells, and of number of flagella per cell), with the current specimen possibly poorer than others, even within the same subspecies [19,38]. However, this variation might be only apparent, considering that different methods can have differential sensitivity (e.g., in the current study negative stain was not employed) and that we did not perform a quantitative analysis of the flagellar abundance.

In any case, we underline that the precise function(s) of flagella in "*Ca*. Trichorickettsia mobilis" and in *Rickettsiales* in general still need(s) to be elucidated. Despite being absent in several long-time studied representatives (e.g., *Rickettsia*, *Orientia*, *Anaplasma*, *Ehrlichia*, *Wolbachia*), it has become evident that flagella [17,23,47] or at least flagellar genes [24,27,46,67,68] are present in many *Rickettsiales*. They are now considered an ancestral trait [24–26,69], independently lost in many extant lineages, possibly concurrently with the adaptation to terrestrial hosts. Indeed, it has been speculated that flagella-driven motility could be important in particular in aquatic environments, for possible intermediate "free-living" phases during horizontal transfer between different hosts [27]. Anyhow, motility might be also involved in as-yet-unexplored stages of the life cycle of "terrestrial" *Rickettsiales* in vertebrate hosts [24]. Alternative roles have been proposed for flagella in *Rickettsiales* [23], in particular as a potential secretion system [24,28]. Indeed, they share a common type III secretion system component with the injectisome [70], which is important in many bacteria for delivering effectors mediating the interaction with host cells. Thus, the flagellum may actually add up to the already known repertoire of apparatuses for delivering effectors to the hosts in *Rickettsiales* [71]. It has also been proposed that the flagellum could be directly mediating the interaction with host cells in the *Rickettsiales* bacterium "*Ca*. Midichloria" [28], with potential analogies with other *Alphaproteobacteria* [72].

In this context of elucidating the role of flagella in *Rickettsiales*, the case of "*Ca*. Trichorickettsia mobilis" is highly interesting for several reasons. First, to our knowledge, its flagellated members are the only ones for which actual motility due to flagella has been observed among *Rickettsiales*. Second, the fact that flagella were observed only in certain strains and subspecies (see references [19,38] and present study) is quite a relevant aspect that still needs to be elucidated, namely whether this is due to the presence/absence of the respective genetic repertoire, or by other causes. For example, it could be speculated that a contingent expression could be triggered only in some conditions, possibly related to host species and host subcellular compartment. In this regard, it is worth noting that the observed presence of flagella seems to correlate more with the bacterial phylogeny, rather than with other features, which might be considered in favour of a genetic explanation. However, any conclusions on this point are clearly premature, and further targeted studies, including in particular genome sequencing, would be necessary to address it. Last but not least, the interest in studying flagella in "*Ca*. Trichorickettsia mobilis" resides in its phylogenetic proximity with *Rickettsia* [17], which includes some of the most medically relevant, and thus deeply studied, *Rickettsiales* [71,73,74]. Investigations on "*Ca*. Trichorickettsia

mobilis", especially but not exclusively focused on flagella, may provide relevant insights on the evolutionary origin of *Rickettsia*, in particular for what concerns the adaptation to terrestrial metazoan hosts, such as arthropods and vertebrates.

In sum, due to the features exposed above, in our view, "*Ca*. Trichorickettsia mobilis" may represent an ideal system to study flagella and their regulation in *Rickettsiales*, from a functional and evolutionary perspective. Thus, we call for further investigations on this bacterium and its interaction with host organisms, including host species-dependent variability, and with a special focus on the role of flagella.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121032/s1, Figure S1: Fluorescence in situ hybridisation pictures of *Pseudokeronopsis* cfr. *erythrina* PSqRJ01.

Author Contributions: Conceptualization, M.C., V.S., M.V.X.S., L.M. and G.P.; formal analysis, M.C. and V.S.; investigation, M.V.X.S., M.C. and L.M.; resources, G.P.; data curation, M.V.X.S., M.C., L.G. and L.M.; writing—original draft preparation, M.C., V.S. and L.M.; writing—review and editing, G.P., M.V.X.S. and L.G.; visualization, L.M. and V.S.; supervision, M.C., L.M. and G.P.; project administration, G.P.; funding acquisition, G.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Community's H2020 Programme H2020-MSCA-RISE 2019: grant agreement n° 872767. The present study was also supported by the Fondazione Cassa di Risparmio di Pistoia e Pescia—giovani@ricercascientifica2019 fellowship (n° 2019.0380).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The sequences newly obtained in this study were deposited to the NCBI GenBank repository, under the following accession numbers: combined partial 18S rRNA gene, ITS and partial 28S rRNA gene of Pseudokeronopsis cfr. erythrina PSqRJ01 (ON331772), "Candidatus Trichorickettsia mobilis subsp. hyperinfectiva" endosymbiont of Pseudokeronopsis cfr. erythrina PSqRJ01 (ON355343).

Acknowledgments: The authors would like to thank Franco Verni and Claudia Vannini for the support in TEM preparation and observation, Simone Gabrielli for figure-editing support, E. Mugnaioli (CISUP) and P. Lucchesi (CIME) for the assistance with HR FEG-TEM observation, and Katrin Grosser for the support in molecular biology experiments.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Husnik, F.; Tashyreva, D.; Boscaro, V.; George, E.E.; Lukeš, J.; Keeling, P.J. Bacterial and Archaeal Symbioses with Protists. *Curr. Biol.* **2021**, *31*, R862–R877. [CrossRef] [PubMed]
2. Nowack, E.C.M.; Melkonian, M. Endosymbiotic Associations within Protists. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2010**, *365*, 699–712. [CrossRef] [PubMed]
3. Dziallas, C.; Allgaier, M.; Monaghan, M.T.; Grossart, H.-P. Act Together—Implications of Symbioses in Aquatic Ciliates. *Front. Microbiol.* **2012**, *3*, 288. [CrossRef]
4. Serra, V.; Gammuto, L.; Nitla, V.; Castelli, M.; Lanzoni, O.; Sassera, D.; Bandi, C.; Sandeep, B.V.; Verni, F.; Modeo, L.; et al. Morphology, Ultrastructure, Genomics, and Phylogeny of *Euplotes Vanleeuwenhoeki* Sp. Nov. and Its Ultra-Reduced Endosymbiont "*Candidatus* Pinguicoccus Supinus" Sp. Nov. *Sci. Rep.* **2020**, *10*, 20311. [CrossRef] [PubMed]
5. Castelli, M.; Sassera, D.; Petroni, G. Biodiversity of "Non-Model" *Rickettsiales* and Their Association with Aquatic Organisms. In *Rickettsiales*; Thomas, S., Ed.; Springer: Berlin/Heidelberg, Germany, 2016; pp. 59–91.
6. Collingro, A.; Köstlbacher, S.; Mussmann, M.; Stepanauskas, R.; Hallam, S.J.; Horn, M. Unexpected Genomic Features in Widespread Intracellular Bacteria: Evidence for Motility of Marine Chlamydiae. *ISME J.* **2017**, *11*, 2334–2344. [CrossRef]
7. Hugoson, E.; Guliaev, A.; Ammunét, T.; Guy, L. Host Adaptation in Legionellales Is 1.9 Ga, Coincident with Eukaryogenesis. *Mol. Biol. Evol.* **2022**, *39*, msac037. [CrossRef]
8. Watanabe, K.; Nakao, R.; Fujishima, M.; Tachibana, M.; Shimizu, T.; Watarai, M. Ciliate *Paramecium* Is a Natural Reservoir of *Legionella pneumophila*. *Sci. Rep.* **2016**, *6*, 24322. [CrossRef]
9. Richards, A.M.; Von Dwingelo, J.E.; Price, C.T.; Abu Kwaik, Y. Cellular Microbiology and Molecular Ecology of *Legionella*-Amoeba Interaction. *Virulence* **2013**, *4*, 307–314. [CrossRef] [PubMed]

10. Mungroo, M.R.; Siddiqui, R.; Khan, N.A. War of the Microbial World: *Acanthamoeba* Spp. Interactions with Microorganisms. *Folia Microbiol.* **2021**, *66*, 689–699. [CrossRef]
11. Wang, Z.; Wu, M. Comparative Genomic Analysis of *Acanthamoeba* Endosymbionts Highlights the Role of Amoebae as a "Melting Pot" Shaping the *Rickettsiales* Evolution. *Genome Biol. Evol.* **2017**, *9*, 3214–3224. [CrossRef]
12. Barker, J.; Brown, M.R. Trojan Horses of the Microbial World: Protozoa and the Survival of Bacterial Pathogens in the Environment. *Microbiology* **1994**, *140 Pt 6*, 1253–1259. [CrossRef] [PubMed]
13. Watanabe, K.; Higuchi, Y.; Shimmura, M.; Tachibana, M.; Fujishima, M.; Shimizu, T.; Watarai, M. Peculiar *Paramecium* Peculiar Hosts Fail to Establish a Stable Intracellular Relationship with *Legionella pneumophila*. *Front. Microbiol.* **2020**, *11*, 596731. [CrossRef] [PubMed]
14. Fujishima, M.; Kodama, Y. Mechanisms for Establishing Primary and Secondary Endosymbiosis in *Paramecium*. *J. Eukaryot. Microbiol.* **2022**, *69*, e12901. [CrossRef] [PubMed]
15. Siegmund, L.; Schweikert, M.; Fischer, M.S.; Wöstemeyer, J. Bacterial Surface Traits Influence Digestion by Tetrahymena Pyriformis and Alter Opportunity to Escape from Food Vacuoles. *J. Eukaryot. Microbiol.* **2018**, *65*, 600–611. [CrossRef] [PubMed]
16. Siegmund, L.; Burmester, A.; Fischer, M.S.; Wöstemeyer, J. A Model for Endosymbiosis: Interaction between Tetrahymena Pyriformis and Escherichia Coli. *Eur. J. Protistol.* **2013**, *49*, 552–563. [CrossRef] [PubMed]
17. Vannini, C.; Boscaro, V.; Ferrantini, F.; Benken, K.A.; Mironov, T.I.; Schweikert, M.; Görtz, H.-D.; Fokin, S.I.; Sabaneyeva, E.V.; Petroni, G. Flagellar Movement in Two Bacteria of the Family Rickettsiaceae: A Re-Evaluation of Motility in an Evolutionary Perspective. *PLoS ONE* **2014**, *9*, e87718. [CrossRef]
18. Mironov, T.; Sabaneyeva, E. A Robust Symbiotic Relationship Between the Ciliate *Paramecium multimicronucleatum* and the Bacterium *Ca.* Trichorickettsia Mobilis. *Front. Microbiol.* **2020**, *11*, 603335. [CrossRef]
19. Sabaneyeva, E.; Castelli, M.; Szokoli, F.; Benken, K.; Lebedeva, N.; Salvetti, A.; Schweikert, M.; Fokin, S.; Petroni, G. Host and Symbiont Intraspecific Variability: The Case of *Paramecium calkinsi* and "*Candidatus* Trichorickettsia Mobilis". *Eur. J. Protistol.* **2018**, *62*, 79–94. [CrossRef]
20. Davison, H.R.; Pilgrim, J.; Wybouw, N.; Parker, J.; Pirro, S.; Hunter-Barnett, S.; Campbell, P.M.; Blow, F.; Darby, A.C.; Hurst, G.D.D.; et al. Genomic Diversity across the Rickettsia and "Candidatus Megaira" Genera and Proposal of Genus Status for the Torix Group. *Nat. Commun.* **2022**, *13*, 2630. [CrossRef]
21. Blanton, L.S. The Rickettsioses: A Practical Update. *Infect. Dis. Clin. N. Am.* **2019**, *33*, 213–229. [CrossRef]
22. Dumler, J.S.; Walker, D.H. Rickettsiales. In *Bergey's Manual of Systematics of Archaea and Bacteria*; Wiley: Hoboken, NJ, USA, 2015; p. 1.
23. Boscaro, V.; Schrallhammer, M.; Benken, K.A.; Krenek, S.; Szokoli, F.; Berendonk, T.U.; Schweikert, M.; Verni, F.; Sabaneyeva, E.V.; Petroni, G. Rediscovering the Genus *Lyticum*, Multiflagellated Symbionts of the Order *Rickettsiales*. *Sci. Rep.* **2013**, *3*, 3305. [CrossRef] [PubMed]
24. Sassera, D.; Lo, N.; Epis, S.; D'Auria, G.; Montagna, M.; Comandatore, F.; Horner, D.; Peretó, J.; Luciano, A.M.; Franciosi, F.; et al. Phylogenomic Evidence for the Presence of a Flagellum and cbb(3) Oxidase in the Free-Living Mitochondrial Ancestor. *Mol. Biol. Evol.* **2011**, *28*, 3285–3296. [CrossRef] [PubMed]
25. Schön, M.E.; Martijn, J.; Vosseberg, J.; Köstlbacher, S.; Ettema, T.J.G. The Evolutionary Origin of Host Association in the Rickettsiales. *Nat. Microbiol.* **2022**, *7*, 1189–1199. [CrossRef] [PubMed]
26. Castelli, M.; Sabaneyeva, E.; Lanzoni, O.; Lebedeva, N.; Floriano, A.M.; Gaiarsa, S.; Benken, K.; Modeo, L.; Bandi, C.; Potekhin, A.; et al. Deianiraea, an Extracellular Bacterium Associated with the Ciliate *Paramecium*, Suggests an Alternative Scenario for the Evolution of *Rickettsiales*. *ISME J.* **2019**, *13*, 2280–2294. [CrossRef] [PubMed]
27. Schulz, F.; Martijn, J.; Wascher, F.; Lagkouvardos, I.; Kostanjšek, R.; Ettema, T.J.G.; Horn, M. A *Rickettsiales* Symbiont of Amoebae with Ancient Features. *Environ. Microbiol.* **2016**, *18*, 2326–2342. [CrossRef] [PubMed]
28. Floriano, A.M.; Biffignandi, G.B.; Castelli, M.; Olivieri, E.; Clementi, E.; Comandatore, F.; Rinaldi, L.; Opara, M.; Plantard, O.; Palomar, A.M.; et al. The Origin and Evolution of Mitochondrial Tropism in *Midichloria* Bacteria. *bioRxiv* **2022**. [CrossRef]
29. Anesi, A.; Buonanno, F.; di Giuseppe, G.; Ortenzi, C.; Guella, G. Metabolites from the Euryhaline CiliatePseudokeronopsis Erythrina. *Eur. J. Org. Chem.* **2016**, *2016*, 1330–1336. [CrossRef]
30. Guella, G.; Frassanito, R.; Mancini, I.; Sandron, T.; Modeo, L.; Verni, F.; Dini, F.; Petroni, G. Keronopsamides, a New Class of Pigments from Marine Ciliates. *Eur. J. Org. Chem.* **2010**, *2010*, 427–434. [CrossRef]
31. Medlin, L.; Elwood, H.J.; Stickel, S.; Sogin, M.L. The Characterization of Enzymatically Amplified Eukaryotic 16S-like rRNA-Coding Regions. *Gene* **1988**, *71*, 491–499. [CrossRef]
32. Petroni, G.; Dini, F.; Verni, F.; Rosati, G. A Molecular Approach to the Tangled Intrageneric Relationships Underlying Phylogeny in *Euplotes* (Ciliophora, Spirotrichea). *Mol. Phylogenet. Evol.* **2002**, *22*, 118–130. [CrossRef]
33. Rosati, G.; Modeo, L.; Melai, M.; Petroni, G.; Verni, F. A Multidisciplinary Approach to Describe Protists: A Morphological, Ultrastructural, and Molecular Study on *Peritromus kahli* Villeneuve-Brachon, 1940 (Ciliophora, Heterotrichea). *J. Eukaryot. Microbiol.* **2004**, *51*, 49–59. [CrossRef] [PubMed]
34. Boscaro, V.; Fokin, S.I.; Verni, F.; Petroni, G. Survey of *Paramecium duboscqui* Using Three Markers and Assessment of the Molecular Variability in the Genus *Paramecium*. *Mol. Phylogenet. Evol.* **2012**, *65*, 1004–1013. [CrossRef] [PubMed]

35. Vannini, C.; Rosati, G.; Verni, F.; Petroni, G. Identification of the Bacterial Endosymbionts of the Marine Ciliate *Euplotes magnicirratus* (Ciliophora, Hypotrichia) and Proposal of "Candidatus Devosia Euplotis". *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 1151–1156. [CrossRef] [PubMed]
36. Manz, W.; Amann, R.; Ludwig, W.; Wagner, M.; Schleifer, K.-H. Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. *Syst. Appl. Microbiol.* **1992**, *15*, 593–600. [CrossRef]
37. Amann, R.I.; Binder, B.J.; Olson, R.J.; Chisholm, S.W.; Devereux, R.; Stahl, D.A. Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Appl. Environ. Microbiol.* **1990**, *56*, 1919–1925. [CrossRef] [PubMed]
38. Modeo, L.; Salvetti, A.; Rossi, L.; Castelli, M.; Szokoli, F.; Krenek, S.; Serra, V.; Sabaneyeva, E.; Di Giuseppe, G.; Fokin, S.I.; et al. Trichorickettsia Mobilis", a Bacterium, Can Be Transiently Transferred from the Unicellular Eukaryote to the Planarian. *PeerJ* **2020**, *8*, e8977. [CrossRef] [PubMed]
39. Westram, R.; Bader, K.; Prüsse, E.; Kumar, Y.; Meier, H.; Glöckner, F.O.; Ludwig, W. ARB: A Software Environment for Sequence Data. In *Handbook of Molecular Microbial Ecology I: Metagenomics and Complementary Approaches*; Wiley-Blackwell: Hoboken, NJ, USA, 2011; pp. 399–406.
40. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
41. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More Models, New Heuristics and Parallel Computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef] [PubMed]
42. Guindon, S.; Gascuel, O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.* **2003**, *52*, 696–704. [CrossRef] [PubMed]
43. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef] [PubMed]
44. Chen, X.; Clamp, J.C.; Song, W. Phylogeny and Systematic Revision of the Family Pseudokeronopsidae (Protista, Ciliophora, Hypotricha), with Description of a New Estuarine Species of Pseudokeronopsis. *Zool. Scr.* **2011**, *40*, 659–671. [CrossRef]
45. Yurchenko, T.; Ševčíková, T.; Přibyl, P.; El Karkouri, K.; Klimeš, V.; Amaral, R.; Zbránková, V.; Kim, E.; Raoult, D.; Santos, L.M.A.; et al. A Gene Transfer Event Suggests a Long-Term Partnership between Eustigmatophyte Algae and a Novel Lineage of Endosymbiotic Bacteria. *ISME J.* **2018**, *12*, 2163–2175. [CrossRef] [PubMed]
46. Martijn, J.; Schulz, F.; Zaremba-Niedzwiedzka, K.; Viklund, J.; Stepanauskas, R.; Andersson, S.G.E.; Horn, M.; Guy, L.; Ettema, T.J.G. Single-Cell Genomics of a Rare Environmental Alphaproteobacterium Provides Unique Insights into Rickettsiaceae Evolution. *ISME J.* **2015**, *9*, 2373–2385. [CrossRef] [PubMed]
47. Lanzoni, O.; Sabaneyeva, E.V.; Modeo, L.; Castelli, M.; Lebedeva, N.; Verni, F.; Schrallhammer, M.; Potekhin, A.; Petroni, G. Diversity and Environmental Distribution of the Cosmopolitan Endosymbiont "*Candidatus* Megaira". *Sci. Rep.* **2019**, *9*, 1179. [CrossRef] [PubMed]
48. Castelli, M.; Serra, V.; Senra, M.V.X.; Basuri, C.K.; Soares, C.A.G.; Fokin, S.I.; Modeo, L.; Petroni, G. The Hidden World of *Rickettsiales* Symbionts: "*Candidatus* Spectririckettsia Obscura," a Novel Bacterium Found in Brazilian and Indian *Paramecium caudatum*. *Microb. Ecol.* **2019**, *77*, 748–758. [CrossRef]
49. Muñoz-Gómez, S.A.; Hess, S.; Burger, G.; Lang, B.F.; Susko, E.; Slamovits, C.H.; Roger, A.J. An Updated Phylogeny of the Reveals That the Parasitic and Have Independent Origins. *Elife* **2019**, *8*, e42535. [CrossRef]
50. Kim, M.; Oh, H.-S.; Park, S.-C.; Chun, J. Towards a Taxonomic Coherence between Average Nucleotide Identity and 16S rRNA Gene Sequence Similarity for Species Demarcation of Prokaryotes. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 346–351. [CrossRef]
51. Mironov, T.; Yakovlev, A.; Sabaneyeva, E. Together Forever: Inseparable Partners of the Symbiotic System *Paramecium multimicronucleatum*/*Ca.* Trichorickettsia Mobilis. *Symbiosis* **2022**, *87*, 19–30. [CrossRef]
52. Hess, S. Description of *Hyalodiscus flabellus* Sp. Nov. (Vampyrellida, Rhizaria) and Identification of Its Bacterial Endosymbiont, "*Candidatus* Megaira Polyxenophila" (*Rickettsiales*, *Alphaproteobacteria*). *Protist* **2017**, *168*, 109–133. [CrossRef]
53. George, E.E.; Husnik, F.; Tashyreva, D.; Prokopchuk, G.; Horák, A.; Kwong, W.K.; Lukeš, J.; Keeling, P.J. Highly Reduced Genomes of Protist Endosymbionts Show Evolutionary Convergence. *Curr. Biol.* **2020**, *30*, 925–933.e3. [CrossRef]
54. Boscaro, V.; Manassero, V.; Keeling, P.J.; Vannini, C. Single-Cell Microbiomics Unveils Distribution and Patterns of Microbial Symbioses in the Natural Environment. *Microb. Ecol.* **2022**. [CrossRef] [PubMed]
55. Boscaro, V.; Husnik, F.; Vannini, C.; Keeling, P.J. Symbionts of the Ciliate *Euplotes*: Diversity, Patterns and Potential as Models for Bacteria-Eukaryote Endosymbioses. *Proc. Biol. Sci.* **2019**, *286*, 20190693. [CrossRef]
56. Berger, H. *Monograph of the Urostyloidea (Ciliophora, Hypotricha)*; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2007; ISBN 9781402052736.
57. Li, J.; Xu, K. Morphology and Taxonomy of *Pseudokeronopsis rubra* (Ehrenberg, 1836) and *Pseudokeronopsis parasongi* Sp. Nov. (Ciliophora, Hypotrichia, Urostylida) from the Yellow Sea. *Eur. J. Protistol.* **2020**, *76*, 125737. [CrossRef]
58. Li, J.; Zhan, Z.; Xu, K. Systematics and Molecular Phylogeny of the Ciliate Genus *Pseudokeronopsis* (Ciliophora, Hypotrichia). *J. Eukaryot. Microbiol.* **2017**, *64*, 850–872. [CrossRef]
59. Chen, X.; Li, J.; Xu, K. Insights into the Phylogeny of Three Systematically Controversial Subfamilies of Urostylid Ciliates Based on rDNA. *Zool. Scr.* **2021**, *50*, 383–395. [CrossRef]

60. Xu, Y.; Shen, Z.; Gentekaki, E.; Xu, J.; Yi, Z. Comparative Transcriptome Analyses during the Vegetative Cell Cycle in the Mono-Cellular Organism *Pseudokeronopsis erythrina* (Alveolata, Ciliophora). *Microorganisms* **2020**, *8*, 108. [CrossRef] [PubMed]
61. Boscaro, V.; Carducci, D.; Barbieri, G.; Senra, M.V.X.; Andreoli, I.; Erra, F.; Petroni, G.; Verni, F.; Fokin, S.I. Focusing on Genera to Improve Species Identification: Revised Systematics of the Ciliate *Spirostomum*. *Protist* **2014**, *165*, 527–541. [CrossRef] [PubMed]
62. Melekhin, M.; Yakovleva, Y.; Lebedeva, N.; Nekrasova, I.; Nikitashina, L.; Castelli, M.; Mayén-Estrada, R.; Romanovich, A.E.; Petroni, G.; Potekhin, A. Cryptic Diversity in Revealed with a Polyphasic Approach. *Microorganisms* **2022**, *10*, 974. [CrossRef]
63. Obert, T.; Vd'ačný, P. Delimitation of Five Astome Ciliate Species Isolated from the Digestive Tube of Three Ecologically Different Groups of Lumbricid Earthworms, Using the Internal Transcribed Spacer Region and the Hypervariable D1/D2 Region of the 28S rRNA Gene. *BMC Evol. Biol.* **2020**, *20*, 37. [CrossRef]
64. Zhang, T.; Shao, C.; Zhang, T.; Song, W.; Vd'ačný, P.; Al-Farraj, S.A.; Wang, Y. Multi-Gene Phylogeny of the Ciliate Genus *Trachelostyla* (Ciliophora, Hypotrichia), With Integrative Description of Two Species, *Trachelostyla multinucleata* Spec. Nov. and T. Pediculiformis (Cohn, 1866). *Front. Microbiol.* **2022**, *12*, 775570. [CrossRef]
65. Shazib, S.U.A.; Vd'ačný, P.; Kim, J.H.; Jang, S.W.; Shin, M.K. Molecular Phylogeny and Species Delimitation within the Ciliate Genus *Spirostomum* (Ciliophora, Postciliodesmatophora, Heterotrichea), Using the Internal Transcribed Spacer Region. *Mol. Phylogenet. Evol.* **2016**, *102*, 128–144. [CrossRef] [PubMed]
66. Belart, P.; Habib, R.; Raposo, D.; Clemente, I.; Alves Martins, M.V.; Frontalini, F.; Figueiredo, M.S.L.; Lorini, M.L.; Laut, L. Seasonal Dynamics of Benthic Foraminiferal Biocoenosis in the Tropical Saquarema Lagoonal System (Brazil). *Estuaries Coast.* **2019**, *42*, 822–841. [CrossRef]
67. Carrier, T.J.; Leigh, B.A.; Deaker, D.J.; Devens, H.R.; Wray, G.A.; Bordenstein, S.R.; Byrne, M.; Reitzel, A.M. Microbiome Reduction and Endosymbiont Gain from a Switch in Sea Urchin Life History. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2022023118. [CrossRef]
68. Kwan, J.C.; Schmidt, E.W. Bacterial Endosymbiosis in a Chordate Host: Long-Term Co-Evolution and Conservation of Secondary Metabolism. *PLoS ONE* **2013**, *8*, e80822. [CrossRef] [PubMed]
69. Castelli, M.; Nardi, T.; Gammuto, L.; Bellinzona, G.; Sabaneyeva, E.; Potekhin, A.; Serra, V.; Petroni, G.; Sassera, D. Host Association and Intracellularity Evolved Multiple Times Independently in the *Rickettsiales*. *bioRxiv* **2022**. [CrossRef]
70. Diepold, A.; Armitage, J.P. Type III Secretion Systems: The Bacterial Flagellum and the Injectisome. *Philos. Trans. R. Soc. B Biol. Sci.* **2015**, *370*, 20150020. [CrossRef]
71. Gillespie, J.J.; Kaur, S.J.; Rahman, M.S.; Rennoll-Bankert, K.; Sears, K.T.; Beier-Sexton, M.; Azad, A.F. Secretome of Obligate Intracellular Rickettsia. *FEMS Microbiol. Rev.* **2015**, *39*, 47–80.
72. Eicher, S.C.; Dehio, C. *Bartonella* Entry Mechanisms into Mammalian Host Cells. *Cell. Microbiol.* **2012**, *14*, 1166–1173. [CrossRef]
73. Perlman, S.J.; Hunter, M.S.; Zchori-Fein, E. The Emerging Diversity of Rickettsia. *Proc. Biol. Sci.* **2006**, *273*, 2097–2106. [CrossRef]
74. Parola, P.; Paddock, C.D.; Socolovschi, C.; Labruna, M.B.; Mediannikov, O.; Kernif, T.; Abdad, M.Y.; Stenos, J.; Bitam, I.; Fournier, P.-E.; et al. Update on Tick-Borne Rickettsioses around the World: A Geographic Approach. *Clin. Microbiol. Rev.* **2013**, *26*, 657–702. [CrossRef]