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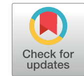
‘*Candidatus* Aquirickettsiella gammari’ (Gammaproteobacteria: Legionellales: Coxiellaceae): A bacterial pathogen of the freshwater crustacean *Gammarus fossarum* (Malacostraca: Amphipoda)☆



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ABSTRACT

Invasive and non-native species can pose risks to vulnerable ecosystems by co-introducing bacterial pathogens. Alternatively, co-introduced bacterial pathogens may regulate invasive population size and invasive traits. We describe a novel candidate genus and species of bacteria (‘*Candidatus* Aquirickettsiella gammari’) found to infect *Gammarus fossarum*, from its native range in Poland. The bacterium develops intracellularly within the haemocytes and cells of the musculature, hepatopancreas, connective tissues, nervous system and gonad of the host. The developmental cycle of ‘*Candidatus* Aquirickettsiella gammari’ includes an elementary body (496.73 nm ± 37.56 nm in length, and 176.89 nm ± 36.29 nm in width), an elliptical, condensed spherical stage (737.61 nm ± 44.51 nm in length and 300.07 nm ± 44.02 nm in width), a divisional stage, and a spherical initial body (1397.59 nm ± 21.26 nm in diameter). We provide a partial genome for ‘*Candidatus* Aquirickettsiella gammari’, which clades phylogenetically alongside environmental 16S rRNA sequences from aquatic habitats, and bacterial symbionts from aquatic isopods (*Asellus aquaticus*), grouping separately from the *Rickettsiella*, a genus that includes bacterial pathogens of terrestrial insects and isopods.

Increased understanding of the diversity of symbionts carried by *G. fossarum* identifies those that might regulate host population size, or those that could pose a risk to native species in the invasive range. Identification of ‘*Candidatus* Aquirickettsiella gammari’ and its potential for adaptation as a biological control agent is explored.

1. Introduction

The Prokaryota includes the diverse group of bacteria (Hugenholtz, 2002; Logares et al., 2014) that are found in a wide range of environments (from ice-sheets to volcanoes), within a diversity of hosts (from humans to protists) and are considered one of the most ancient lineages of life (3–4 Gya) (Poole et al., 1999; DeLong and Pace, 2001). Many bacterial taxa have adapted to survive through colonisation of a host, acting either as parasites or mutualists to survive (Bhavsar et al., 2007; Chow et al., 2010). The evolutionary systematics of bacterial taxa is being revolutionised through wider application of DNA sequencing techniques and development of improved phylogenetic tools to resolve their position within the tree of life (Konstantinidis and Tiedje, 2007).

Some bacterial taxa reside within the cells of their host, utilising resources within the cell for their own division and development. One such intracellular bacterium is the well-known *Chlamydia trachomatis*, a common sexually transmitted disease in humans (Campbell et al., 1987; Stephens et al., 1998). Several others are either medically or

☆ Depositories: NCBI (gene sequence data), Registry of Aquatic Pathology, Cefas, UK (original slides, TEM blocks and ethanol fixed material).

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economically important, resulting in diseases that cause significant healthcare costs, or cultured species yield losses (Pospischil et al., 2002). Others are interesting from a biodiversity and wildlife pathogen perspective (Duron et al., 2015).

The Genus *Rickettsiella* (Philip, 1956) comprises an important taxonomic group of arthropod-infecting bacteria. *Rickettsiella* resides within the family Coxiellaceae (Garrity et al., 2007) along with the genera: *Aquicella* (Santos et al., 2003); '*Candidatus* Berkiella' (Mehari et al., 2015); *Coxiella* (Philip, 1948); and *Diplorickettsia* (Mediannikov et al., 2010). Many of these genera include pathogens of invertebrates. Type description of *Rickettsiella* came from *Rickettsiella popilliae*, a species that infects the fat body of *Popillia japonica* (Japanese beetle) and two species of June beetle (*Phyllophaga*) (Dutky and Gooden, 1952; Philip, 1956). Despite a range of species now included in the *Rickettsiella*, DNA sequence data and phylogenetic analysis are lacking and is required to support their inclusion (example: *Rickettsiella chironomi*) (Philip, 1956).

The *Rickettsiella* are thought to have diverged from *Coxiella* ~350 million years ago (Cordaux et al., 2007) and currently ~9 *Rickettsiella* species are considered adequately described using genetic, morphological and pathological information. All are obligate intracellular bacterial pathogens of arthropods. *Rickettsiella agriotidis* (Leclerque et al., 2011) (host: *Agriotes* sp.), *Rickettsiella pyronotae* (Kleespies et al., 2011) (host: *Pyronota* spp.), *Rickettsiella costelytrae* (Leclerque et al., 2012) (host: *Costelytrae zealandica*) [*R. pyronotae* and *R. costelytrae* now identified as synonyms of *R. popilliae* (Leclerque et al., 2012] and *Rickettsiella melolonthae* (Krieg, 1955) (host: *Melolontha melolontha*) all infect the cells of beetles (Insecta: Coleoptera). *Rickettsiella grylli* (Roux et al., 1997) (host: *Gryllus bimaculatus*) infects the cells of crickets (Insecta: Orthoptera). *Rickettsiella viridis* (Tsuchida et al., 2014) (host: *Acyrthosiphon pisum*) infects the cells of aphids (Intecta: Hemiptera). *Rickettsiella porcellionis* (Kleespies et al., 2014) (host: *Porcellio scaber*) and *Rickettsiella armadillidii* (Cordaux et al., 2007) (host: *Armadillidium vulgare*) infect cells of isopods (Crustacea: Isopoda) and are suggested to be synonymous in a single taxon: *Rickettsiella isopodorum*, based on genomic evidence (Kleespies et al., 2014). Further discoveries have revealed a separate taxon referred to as '*R. grylli* from an isopod' (a name that requires more accurate taxonomic assessment), which also has genomic data (Kleespies et al., 2014; Wang and Chandler, 2016). To date, all described taxa within the *Rickettsiella* are from terrestrial hosts, apart from *Rickettsiella tipulae* (Leclerque and Kleespies, 2008), which infects the crane fly *Tipula paludosa*; an insect with a semi-aquatic life history.

Several other *Rickettsiella*-like taxa have been described to infect the cells of aquatic hosts, but their description is only based on morphological information. These include those infecting the aquatic crustaceans: *Carcinus mediterraneus* (Bonami and Pappalardo, 1980); *Paralithoides platypus* (Johnson, 1984); *Cherax quadricarinatus* (Romero et al., 2000); *Eriocheir sinensis* (Wang and Gu, 2002); three species of penaeid shrimp (Anderson et al., 1987; Brock, 1988; Krol et al., 1991); and two amphipods, *Gammarus pulex* (Larsson, 1982) and *Crangonyx floridanus* (Federici et al., 1974). Over 100 16S rRNA gene sequence accessions exist within online databases for bacterial isolates linked to the *Rickettsiella*, and these include taxa infecting a wide diversity of arthropod hosts, including isolates from aquatic hosts (NCBI). An example from an aquatic host includes an isolate from the aquatic isopod *Asellus aquaticus* (NCBI: AY447041), that lacks morphological and ultrastructural information.

Rickettsiella spp. are considered to have a slow developmental cycle, which involves initially entering a host cell through phagocytosis, dividing within a vacuole, and eventually lysing the cell before completing its lifecycle (Cordaux et al., 2007). In detail, small, dense elementary bodies are first phagocytosed by the host cell, prior to their enlargement (Kleespies et al., 2014). These enlarge into spherical bodies, which in insects at least, often contain a crystalline substance that has not yet been observed in those *Rickettsiella* infecting crustaceans (Kleespies et al., 2014). Finally, these enlarged cells condense and divide before condensing further into infective stage elementary bodies (Kleespies et al., 2014).

Rickettsiella spp. often cause disease in their host. Some have been associated with clinical signs, leading to descriptions such as "Blue Disease" or "Milky Disease" (Dutky and Gooden, 1952; Kleespies et al., 2011). In insects, disease often results in an iridescent appearance to the infected tissues (Dutky and Gooden, 1952; Kleespies et al., 2011). In crustaceans, clinical signs include an opaque white appearance of fluids and intersegmental membranes (Vago et al., 1970; Federici et al., 1974). In all cases, bacterial colonies are observed in the cytoplasm of host cells, causing displacement of organelles and cellular hypertrophy (Federici et al., 1974; Kleespies et al., 2014). Although genomic information is not available for many taxa, a full genome sequence is available for an *R. grylli* isolate from an isopod (Leclerque, 2008), and a genome is available for *R. isopodorum*; along with several others from the *Coxiellaceae* but outside the *Rickettsiella* (Seshadri et al., 2003; Mehari et al., 2015).

As part of a disease survey of the amphipod *Gammarus fossarum* for pathogens and symbionts, we discovered infection and disease associated with a novel bacterium. We utilise high throughput sequencing data to construct a partial genome of the pathogen and provide complementary information obtained from transmission electron microscopy and histopathology to describe a novel genus and species, '*Candidatus* Aquirickettsiella gammari', as a candidate sister genus to the *Rickettsiella*. The pathogen infects the cytoplasm of circulating haemocytes and cells of the gonad, nerve, hepatopancreas, connective tissues and musculature of the amphipod and may have future applicability as a control agent for invasive and non-native *G. fossarum*.

2. Materials and methods

2.1. Animal collection

Gammarus fossarum (n = 140) were collected from the Bzura River in Łódź (Łagiewniki), Poland (N51.824829, E19.459828) in June 2015. One hundred and twenty-seven individuals were fixed for histology on site while 13 were transported live to the University of Łódź for dissection. Dissection involved initial cooling to anaesthetise the individual before removing and dividing the hepatopancreas, gut and muscle tissue for fixation for molecular diagnostics (96% EtOH), histology [Davidson's freshwater fixative (Hopwood, 1996)] and, transmission electron microscopy (TEM) (2.5% glutaraldehyde in sodium cacodylate buffer) according to protocols published by our laboratory (Bojko et al., 2015).

2.2. Histopathology and transmission electron microscopy

For histology, whole animals or dissected organs and tissues were initially fixed in Davidson's freshwater fixative for 48 hr. After fixation, the tissues were submerged in 70% ethanol and transported to the Cefas Weymouth Laboratory, UK for histological processing. Specimens were decalcified for 30 min before placement in 70% industrial methylated spirit and transfer to an automated tissue processor (Leica, UK) for wax infiltration. Whole animals, or dissected organs and tissues, were embedded in wax blocks and sectioned at 3 µm before transfer to glass slides. Sections were stained using haematoxylin and alcoholic eosin (H&E) and mounted with a glass coverslip using DPX. All slides were read using standard light microscopy (Nikon E800, Nikon, UK). Digital images were captured using an integrated camera (Leica, UK) and Lucia Image Capture software. For TEM, dissected tissues (muscle and hepatopancreas) were processed and analysed according to Bojko et al. (2015). Digital images were obtained on a Jeol JEM 1400 transmission electron microscope using on-board camera and software (Jeol, UK). These two techniques identified a previously unknown bacterial infection, providing the incentive to apply molecular tools for bacterial

systematics.

2.3. DNA extraction, PCR, sequencing, and in-situ hybridisation (ISH)

Ethanol-fixed muscle biopsies from infected amphipods (n = 3) were initially digested using proteinase K (10 mg/ml) in solution with Lifton's Buffer (0.1 M Tris-HCl, 0.5% SDS, 0.1 M EDTA). The solution extracts were analysed for 16S rRNA sequence in a single-round Taq polymerase PCR protocol using the general bacterial 16S primers fD1 and rP2 according to Weisburg et al. (1991). Amplicons (~1900 bp) were excised from the gel and forward and reverse sequenced using the 'eurofinsgenomics' service (www.eurofinsgenomics.eu). This sequence length was not used in the phylogenetic comparison, but rather the larger sequence obtained from metagenomic analysis which shared 100% sequence identity. Each specimen positive for infection via histology provided the same 16S rRNA gene sequence.

The amplicon was also used as an ISH probe upon histological section. The band was isolated and purified using Polyethylene Glycol 8000® Sigma-Aldrich (Lis, 1980), and the purified DNA was digoxigenin (DIG)-labelled using the same PCR conditions above, but with altered reagent concentrations (10 µl colourless buffer, 5 µl $MgCl_2$ solution, 5 µl of PCR DIG labelling mix (Roche), 3 µl template DNA, 1 µl of forward and reverse primers, 0.5 µl of GoTaq Polymerase and 24.5 µl molecular water). The control was produced by amplifying the same 16S rDNA gene using non-labelled standard dNTPs. Products were purified as previous, and the amount of DNA quantified (NanoDrop 1000 Spectrophotometer® Thermo Scientific) and diluted to 1 ng/µl, for a total volume of 50 µl.

The ISH technique presented below is an adaptation from published protocols (Montagnani et al, 2001; Fabioux et al, 2004). Dry tissue sections were dewaxed and rehydrated: Clearene for 5 min (2 times), followed by 100% industrial denatured alcohol (IDA) for 5 min and 70% IDA for another 5 min. Slides were rinsed in 0.1 M TRIS buffer (0.1 M TRIS base, 0.15 M NaCl, adjust the pH to 7.5 adding HCl) and placed in a humid chamber before being covered with 300 µl of 0.3% Triton-X diluted in 0.1 M TRIS buffer (pH 7.5) for 20 min and rinsed with 0.1 M TRIS buffer (pH 7.5). Tissue was covered with Proteinase K (25 µg/ml) in 0.1 M TRIS buffer (pH 7.5) (37 °C) and kept for 20 min at 37 °C to prevent evaporation. Slides were washed in 70% IDA for 3 min and 100% IDA for 3 min before rinsing in SSC (2×) for 1 min while gently agitating (SSC 1×: 0.15 M NaCl and 0.015 M Sodium Citrate). Slides were kept in 0.1 M TRIS buffer (pH 7.5) until the In-Situ hybridization frame seals (BIO-RAD) are duly placed. The DIG labelled probe and the non-labelled probe (control) were both diluted 1:1 with hybridization buffer and added to the slide. After DNA denaturation (94 °C for 6 min), slides were hybridised overnight at 44 °C.

Samples were washed for 10 min with washing buffer (25 ml of SSC 20×, 6 M Urea, 2 mg/L BSA), before a further 2 washes in preheated (38 °C) washing buffer for 10 min. Slides were rinsed with preheated (38 °C) SSC (1×) for 5 min (twice) and with 0.1 M TRIS buffer (pH 7.5) (twice). The blocking step included a solution of 6% dried skimmed milk diluted in 0.1 M TRIS buffer (pH 7.5) for 1 hr and washed with 0.1 M TRIS buffer (pH 7.5) for 5 min (twice). Slides were incubated with 1.5 U/ml of Anti-Digoxigenin-AP Fab fragments (Roche) diluted in 0.1 M TRIS buffer (pH 7.5) for 1 hr in darkness. Excess Anti-DIG-AP was removed. Slides were transferred to 0.1 M TRIS buffer (pH 9.5) for 2 min, and the slide was covered with NBT/BCIP stock solution (Roche) diluted in 0.1 M TRIS buffer (pH 9.5) and incubated in darkness until the first clear signs of blue staining start to appear (~30 min). Slides were washed in 0.1 M TRIS buffer (pH 9.5) for 1 min (twice) and stained with 1% Bismark Brown (6 min) and dehydrated in 70% IDA, 45 s in 100% IDA, then washed twice in Clearene (1 min) prior to coverslip. The ISH probe confirmed infection in haemocytes and cells of the musculature, gill, gonad, hepatopancreas and nerve tissues of the host. Labelling was not detected in uninfected individuals. In some animals, infection was specifically detected as dense inclusions within the cells of the hepatopancreatic tubules.

2.4. Genome sequencing, assembly and annotation

Muscle tissue from an infected *G. fossarum* carcass, initially fixed in 96% ethanol, was prepared for metagenomic analysis using the Illumina MiSeq platform (Illumina, UK). Corresponding histology for this specimen included only '*Candidatus* Aquirickettsiella gammari' pathology, without visible infection/pathologies caused by other bacteria. The specimen was split into 3 sub-samples with 1 ng of DNA from each sub-sample prepared for sequencing by Nextera XT library preparation per manufacturer's protocol (Illumina; www.illumina.com). Libraries were quality and size checked by bioanalyzer (Agilent; www.agilent.com) and quantified by QuantiFluor fluorimeter (Promega, www.promega.com) before being pooled in equimolar concentrations, denatured by sodium hydroxide, and diluted to 10 pM in Illumina HT1 hybridisation buffer for sequencing. Sequencing was done on an Illumina MiSeq system using the MiSeq Reagent Kit v2 (500 cycles). In total, 23,090,904 individual reads were attained (50,178,184 paired reads) from the sequencer and 46,181,808 reads remained after quality-trimming and low quality read removal.

Raw sequences were quality-trimmed using Trimmomatic v0.32 (Bolger et al., 2014) with the following parameters: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Paired-end reads were merged using PEAR v0.9.8 (Zhang et al., 2014) and subsequently assembled *de novo* using metaSPAdes v3.9.0 with default parameters and k-mers lengths of 21, 33, 55, 77, 99 and 127 (Nurk et al., 2016). The resulting contigs were annotated using PROKKA v1.11 (Seemann, 2014) and sequence similarity searches of the predicted protein sequences were carried out using the BLASTX algorithm of DIAMOND v0.9.14 (Buchfink et al., 2015; using the –sensitive parameter) and the NCBI RefSeq Protein database (31/03/2018). Sequence similarity data can be found in Supplementary Table 1. The DIAMOND output was loaded into MEGAN Community Edition v6.5.5 (Huson et al., 2016); using prot_acc2tax-Mar2018X1.abin) and all contigs containing predicted coding sequences of the Order Legionellales were extracted. In addition, Blobplots were generated with Blobtools v1.0 (Laetsch and Blaxter, 2017) using a mapping file, generated by mapping all read-pairs to the *de novo* assembled contigs using bwa (Li and Durbin, 2009) and SAMtools (Li et al., 2009), and sequence similarity hits files generated by blastn (Camacho et al., 2009) and the NCBI nt database (24/07/2017), and DIAMOND and the UniProt Reference Proteomes database (2018-02), respectively (E value cut off: 1e-25). The 'bestsumorder' taxrule was used for computing taxonomy and contigs annotated to Legionellales were extracted and merged with the contigs obtained with DIAMOND/MEGAN above. The final genome assembly and annotation completeness were determined using BUSCO v3.0.1 (Simão et al., 2015) with the Gammaproteobacteria gene dataset (odb9). For comparative analysis, BUSCO analysis was also performed on the genomes of *R. grylli* (Genbank Assembly Accession GCA_000168295.1) and *Legionella* sp. 40-6 (Genbank Assembly accession GCA_001899505.1). The genome assembly was aligned to the genomes of *R. grylli* isolated from an isopod (NCBI Genbank Accession GCA_000168295.1) and *R. isopodorum* (NCBI Genbank Accession number GCA_001881495.1) using MAUVE snapshot_2015-02-13 (Darling et al., 2004) with default parameters. The genome sequence of '*Candidatus* Aquirickettsiella gammari' was deposited in NCBI GenBank under the accession number NMOS00000000.1. The version described in this paper is the first version.

2.5. Phylogenetics

Predicted gene sequences were utilised in combination with available sequence data from NCBI to generate both a Neighbour-Joining (NJ) phylogenetic tree and Maximum-Likelihood (ML) phylogenetic

tree for a concatenated 20-gene phylogeny, and production of a NJ tree and ML tree for bacterial 16S data, using MEGA 7.0.21 (Kumar et al., 2016). In both cases the ML tree topology is used in the respective figures.

The concatenated phylogeny was constructed from 20 end-to-end gene sequences [23S rRNA, 16S rRNA, 50S L1-5, 30S S1-5, DNA Pol III alpha/beta/tau/delta/epsilon subunit, DNA primase, Replicative DNA Helicase (DnaB), DNA Pol I], which are primarily house-keeping/conserved genes for 8 individual bacterial taxa for which data was available, including *Chlamydophila pneumoniae* to root the tree (NCBI Genbank Accession numbers for all the genes used are shown in Supplemental Table 2). Multiple sequence alignments were generated using the ClustalW algorithm with default settings in MEGA 7.0.21, and phylogenetically compared using the Tamura-3 parameter model (Tamura, 1992) of evolution with uniform rate heterogeneity and the complete deletion model selection algorithm to form a final tree using both NJ (genetic distance) (Saitou and Nei, 1987) and ML methods. The NJ method utilised the Tamura-3 parameter model also, with transitions and transversions accounted for, including Gamma distribution with gamma parameter 5.1 and homogenous pattern among lineages. The NJ also used the complete deletion treatment of the data. The clade credibility for both trees was assessed using bootstrap tests with 200 replicates.

The phylogenetic analysis of the 16S rRNA gene utilised various bacterial isolates (36 species in total), including two *Chlamydophila* sp. that acted as an out-group to root the tree. Two trees were constructed, the first using the same ML method as stated above, but with 500 bootstrap replicates and the use of all sites (gaps/missing data). The second method utilised an NJ approach (Saitou and Nei, 1987) in combination with the Jukes and Cantor (1969) algorithm, pairwise deletion model, and uniform rates of heterogeneity, with 500 bootstrap replicates. The ML tree topology is displayed in this manuscript.

3. Results

3.1. Histopathology and ultrastructure of a novel bacterial species

Gammarus fossarum were infected with an intracellular bacterial infection at a prevalence of 37.8%, which is identified herein as '*Candidatus* Aquirickettsiella gammari' (Fig. 1). Externally, infected hosts had a creamy-white appearance due to the heavy burden of bacterial infection, which was iridescent, and included the presence of orange beads running along the carapace. In 14.2% of hosts, the bacterial infection was only apparently located within epithelia cells of the hepatopancreas (Fig. 2), proposing that this may be the initial seat of infection prior to systemic spread. In other cases, '*Candidatus* Aquirickettsiella gammari' was also present within the haemocytes, which were highly hypertrophic and enlarged (Fig. 1a), and cells of the nervous system (Fig. 1b, c), gonad, connective tissues, musculature (Fig. 1d) as well as the hepatopancreas (Fig. 2). In all tissues the bacterial pathogen resulted in a hypertrophic cytoplasm that stained deep purple under H&E. The infection resulted in the aggregation of haemocytes in addition to extreme hypertrophy of all infected tissues (Fig. 1a).

TEM revealed an intracellular bacterium in cells of the hepatopancreas (Fig. 2), the space beneath the sarcolemma of muscle cells (Fig. 3a) and in the cytoplasm of haemocytes (Fig. 3b). The inclusions within the hepatopancreas had a different morphology to those within the connective tissues, primarily the presence of fibrous material within spherical stages, which is not observed in other infected tissues (Fig. 2d). Primary infection may occur within the hepatopancreas epithelial cells, with liberated bacteria potentially being phagocytosed by haemocytes and connective tissue cells. Bacteria with a highly condensed cytoplasm measured 496.73 nm ± 37.56 nm (n = 20) in length, and 176.89 nm ± 36.29 nm in width, contained an electron dense core (Fig. 3c, d) and electron lucent lamella (Fig. 3d). The bacteria apparently developed through four main stages (Fig. 3e–h), but the order of this developmental process is unknown. The putative first stage is the electron dense elementary body (Fig. 3e), followed by an elliptical, condensed spherical stage [737.61 nm ± 44.51 nm (n = 10) in length and 300.07 nm ± 44.02 nm in width (n = 17)], with an electron lucent cytoplasm (Fig. 3f), which then putatively underwent division (Fig. 3g). Spherical initial bodies were the largest stages observed, measuring 1397.59 nm ± 21.26 nm (n = 10) in diameter (Fig. 3h). Federici et al. (1974) utilised specific methods to identify a crystalline cell surface protein layer [S-layer (glyco) proteins] in the bacteria they observed, but this technique was not conducted here so comparison cannot be confidently made. Crystalline inclusion bodies, often observed in insect-infecting *Rickettsiella*, were not observed during infection by '*Candidatus* Aquirickettsiella gammari'.

An in-situ hybridisation (ISH) probe corresponding to the bacterial isolate for which we provide genomic information, was observed to bind to the presence of bacteria within the muscle (Fig. 4a, b), hepatopancreas (Fig. 4c, d), gonad, gill, connective tissues and nerve tissue; suggesting synonymy between the sequenced isolate and the bacteria infecting each tissue type. Denser staining in infected hepatopancreatic epithelial cells provides evidence that these cells contain large accumulations of the bacteria and likely act as the seat of infection prior to spread to other tissues and organs.

3.2. '*Candidatus* Aquirickettsiella gammari' genome sequence and annotation

Sequence assembly produced 29,089 contigs with a minimum length of 200 bp (N50 = 685), accounting for 16,125,808 bp. Annotation analysis using Diamond and Megan identified 55 contigs with high similarity to Legionellales. Blobplot analysis identified a cluster of 39 contigs with high coverage and similarity to Legionellales (Supplemental Fig. 1) and among these contigs were 10 additional sequences that were not classified as Legionellales by Diamond/Megan analysis. Together, a total of 65 contigs representing '*Candidatus* Aquirickettsiella gammari' were found, with lengths ranging from 220 to 149,894 bp and a total partial genomic length of 1,491,410 bp (N50: 73,822 bp).

Alignment of the '*Candidatus* Aquirickettsiella gammari' genome against the complete *R. grylli* genome identified conserved segments (Locally Collinear Blocks; LCBs) along the full reference genome sequence (Fig. 5). Additional comparison to the genome of *R. isopodorum* suggests that this genome has the most LCBs (Fig. 5). Annotation of the '*Candidatus* Aquirickettsiella gammari' genome assembly resulted in the identification of 1386 coding regions of which a total of 996 had homologues that most closely associated with those encoded in the *R. isopodorum* genome isolated from an isopod (Supplementary Table 1). A total of 82.1% of complete BUSCO genes (371 out of 452) specific to Gammaproteobacteria were recovered, increasing to 84.1% if fragmented genes were included. This compared well to the number of complete BUSCO genes recovered from the genomes of *R. grylli* (2 contigs, total length of 1,581,239 bp) and *Legionella* sp. 40-6 (656 contigs, total length of 3,142,726 bp), with values of 80.5% and 69%, respectively.

Three hundred and sixty-nine of the predicted genes encode for hypothetical proteins and have not yet been fully characterised. Sequences for 16S, 23S and 5S rRNA were also featured within the 65 contigs as well as 40 tRNAs (for guiding 19 unique amino acids, excluding selenocysteine and isoleucine) and 1 tmRNA (SsrA) (see NCBI submission: NMOS00000000/PRJNA392245). The genes included on the 65 contigs suggest a wide range of metabolic and physiological capabilities; of interest here are those that may be involved in virulence. These include secretion systems (Vir, Dot, Icm), other Type IV secretion proteins, and conjugal transfer proteins (Tra/Trb), which may aid horizontal gene transfer to conspecifics and host cells. '*Candidatus* Aquirickettsiella gammari' encodes 8 genes that show similarity to Vir-

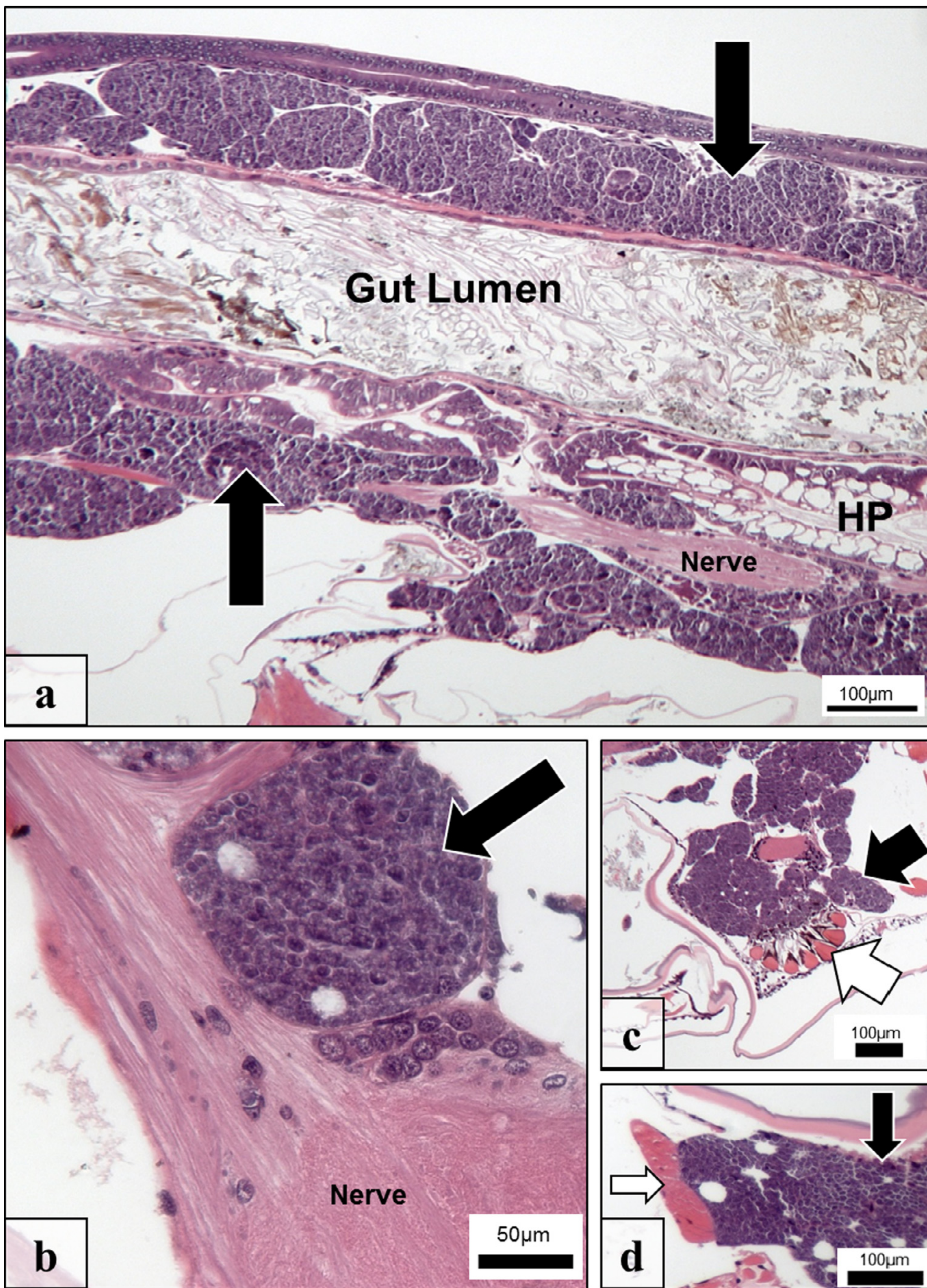

Fig. 1. '*Candidatus* Aquirickettsiella gammari' histopathology in its host, *Gammarus fossarum*. (a) A low magnification histology image of the pereon of an infected *G. fossarum*. The gut lumen and hepatopancreas ('HP') are uninfected with bacteria in this example. The haemolymph (black arrow), nerve tissue ('Nerve') and muscle are all heavily burdened by growing intracellular bacterial plaques that stain deep purple (black arrow). Scale = 100 µm. (b) A detailed histological image of the bacterial pathology (black arrow) upon nerve tissue. The infection forms plaques within the nerve fibres and neurosecretory cells. Scale = 50 µm. (c) The eye (white arrow) and surrounding nerve tissue (black arrow) is infected, possibly resulting in decreased vision. Scale = 100 µm. (d) The muscle (white arrow) sarcolemma is colonised by the bacterial infection and over proliferated (black arrow). Scale = 100 µm.

like proteins, all of which show closest similarity to species outside of the *Rickettsiella*, *Diplorickettsiella* and *Coxiella*, and are more similar to *Legionella* sp. (45–50% similarity), '*Candidatus* Neoehrlichia lotoris' (45.3% similarity), *Sphingopyxis* sp. (38.4% similarity), *Tatlockia micdadei* (57.5%), and *Virgibacillus senegalensis* (73.1% similarity). Ten Dot-like genes primarily showing similarity to *Rickettsiella* sp., and one to *Legionella fairfieldensis* (61.9% similarity). Eight genes that show similarity to Icm-like genes that all show closest similarity to *Rickettsiella isopodorum*. Eighteen genes that are Type-IV-secretion-system-like, primarily with closest similarity to *Rickettsiella isopodorum*; however, 4 show similarity to members of the *Legionella*, *Bartonella* and *Sphingopyxis*. Finally, TraA-like (*Legionella* sp.), TraD-like (*Legionella* sp.) and TrbN-like (*Rickettsiella* sp.) genes are encoded that show closest similarity to the *Legionella* and *Rickettsiella*. Detailed outputs can be found in Supplementary Table 1.

For the most part, the virulence genes encoded by '*Candidatus* Aquirickettsiella gammari' are linked with close relatives in the Coxiellaceae and Legionellales; however, those that seem more closely linked with distant species, such as '*Candidatus* Neoehrlichia lotoris' (a tick-borne disease of humans), *Sphingopyxis* sp. (hardy bacteria that thrive in polluted environments), and *Virgibacillus senegalensis* (a species linked with the human microbiome), may be the result of historic horizontal gene transfers that have contributed to the pathogenicity of '*Candidatus* Aquirickettsiella gammari'.

In addition to genes linked directly with virulence, genes that are involved in the production of toxins may also be linked to the pathology observed in this study and contribute to the declining health of the host. '*Candidatus* Aquirickettsiella gammari' encodes 7 different genes showing closest similarity to 7 different bacterial species that are toxin-like genes, including 6 Type-II-toxin-antitoxin-system genes [RatA

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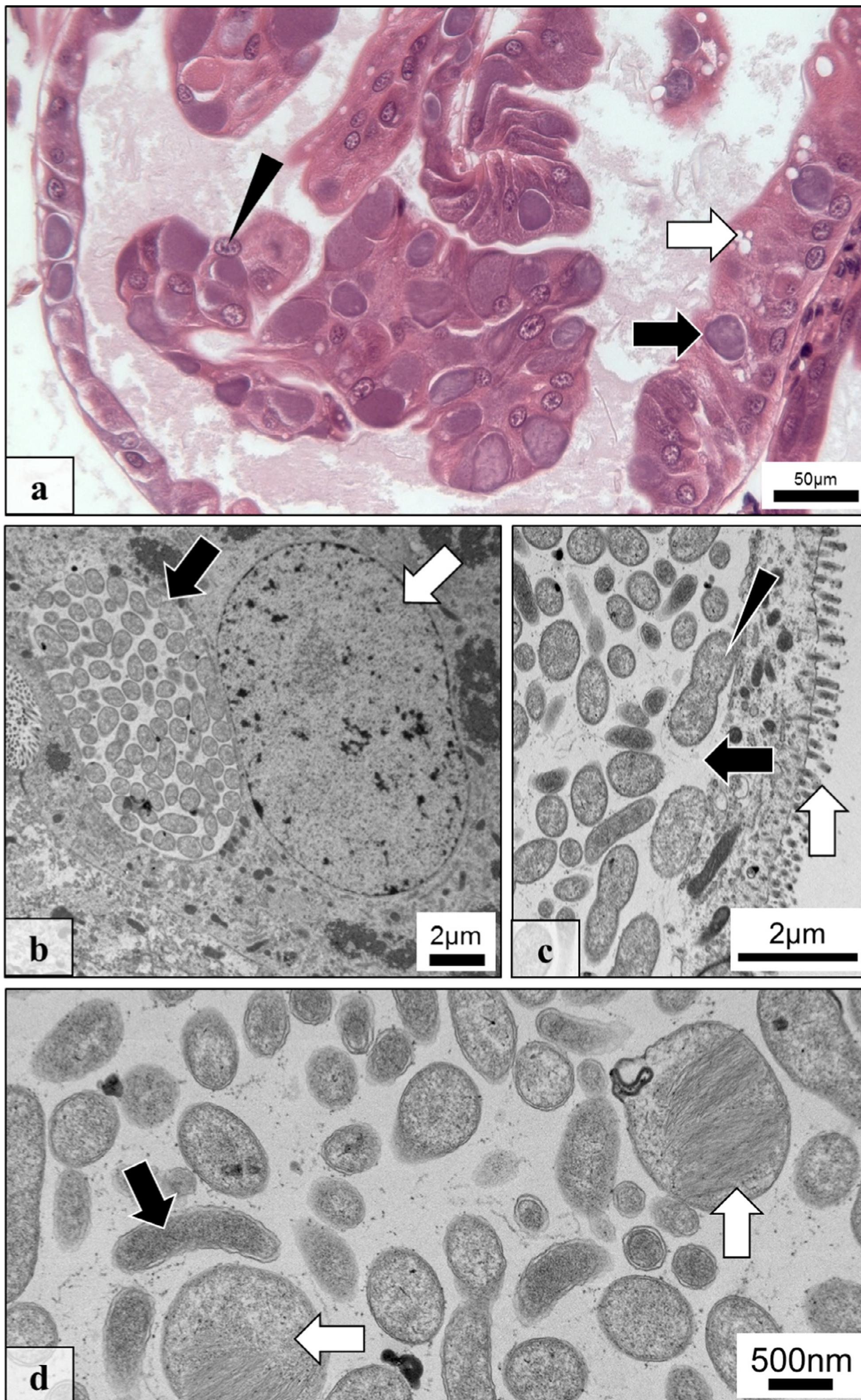

Fig. 2. '*Candidatus* Aquirickettsiella gammari' infecting the hepatopancreas of the host, *Gammarus fossarum*. (a) Histologically derived image of the pathology, where the cytoplasm of alpha and beta cells in the hepatopancreas display intracytoplasmic bacterial infection (black arrow), which does not physically interact with the cytoplasm or nucleus (black triangle). An uninfected cell is indicated with a white arrow. Scale = 50 μm. (b) Transmission electron micrograph of a vacuole containing the unidentified bacteria (black arrow) next to the nucleus (white arrow). Scale = 2 μm. (c) Various bacterial developmental stages, including bacterial division (black triangle). The vacuole is electron lucent (black arrow) and pressing up against the hepatopancreatic villi (white arrow). (d) Elementary bodies (black arrow) and spherical bodies containing fibrous inclusions (white arrow).

(*Rickettsiella isopodorum*: 77.8%); RelE/ParE (*R. grylli*: 79.3%); VapC (*Devosia geojensis*: 68.6%); VapC (*Chlorobium ferrooxidans*: 42.0%); Phd/YefM (*Arboriscoccus pini*: 62.3%); RelE/StbE ('*Candidatus* Glomeribacter gigasporarum': 68.4%)] and a cytolethal distending toxin subunit showing 39.3% similarity to a gene encoded by *Yersinia nurmii*, a species isolated from packaged meat products for human consumption.

3.3. Phylogeny of 'Candidatus Aquirickettsiella gammari'

The 16S rRNA gene of '*Candidatus* Aquirickettsiella gammari' was used to search the NCBI database for similar taxa, determining that the closest known species is a *Rickettsiella*-like bacterium of *Asellus aquaticus* (similarity = 99%; e-value = 0.0) (AY447040) and that the most closely related species with taxonomic description was *R. isopodorum*

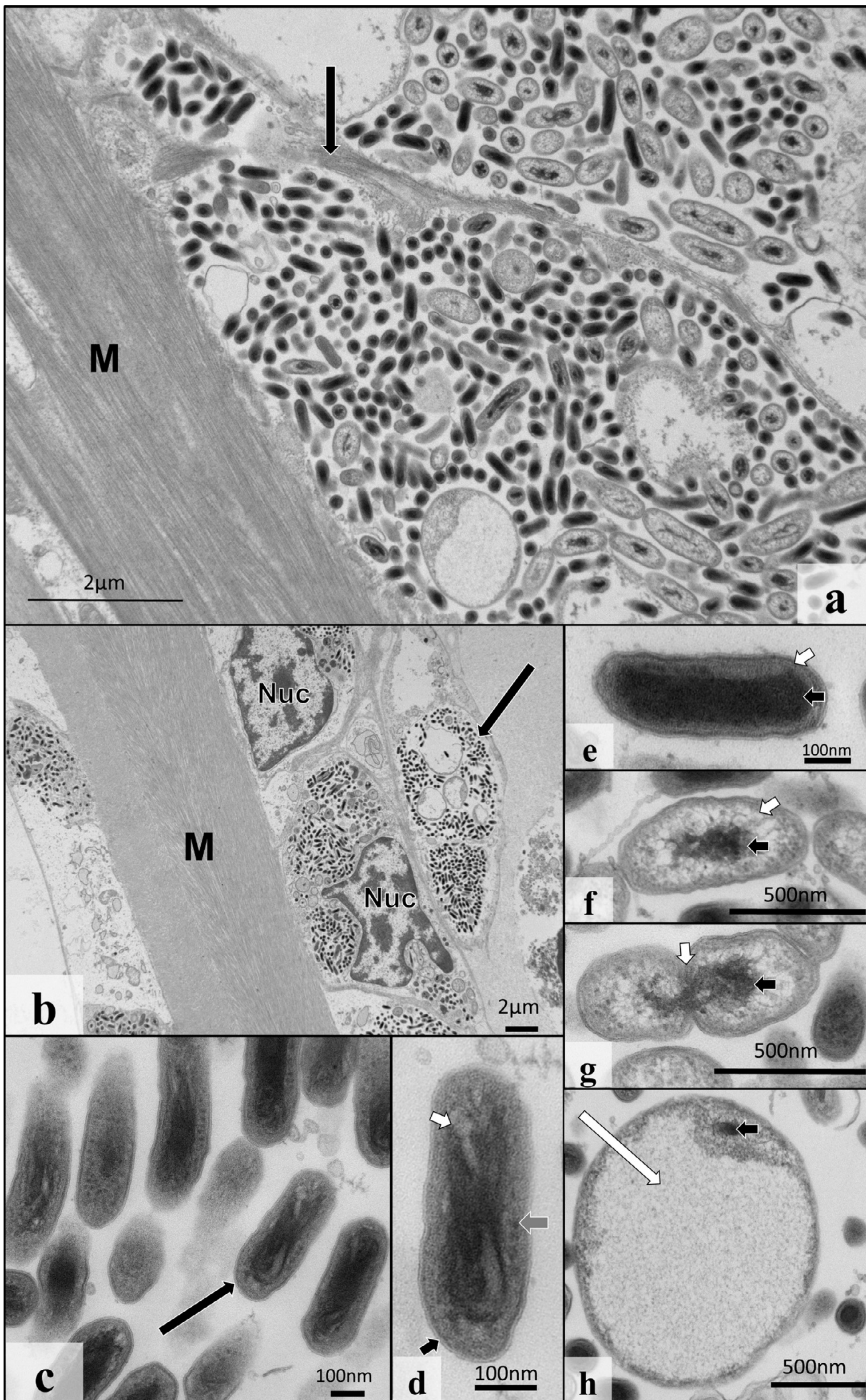

Fig. 3. '*Candidatus* Aquirickettsiella gammari' ultrastructure and development cycle in muscle and haemolymph. (a/b) TEM images of the pathology reveal that the sarcolemma of the muscle ('M') and the haemocytes (nuclei = 'Nuc') are infected with a rickettsia-like organism displaying four developmental stages. Scales = 2 μm. (c) High magnification TEM image of the elementary bodies (black arrow). Scale = 100 nm. (d) The elementary bodies are present with an electron-lucent lamellae (white arrow), condensed, electron dense bodies in the bacterial cytoplasm (grey arrow), a bi-laminar outer membrane (black arrow) and an electron dense core. Scale = 100 nm. The lifecycle of '*Candidatus* Aquirickettsiella gammari' includes images 'e' (condensed elementary body) (scale = 100 nm), 'f' (elliptical condensed sphere stage) (scale = 500 nm), 'g' (division) (scale = 500 nm), and 'h' (spherical body) (scale = 500 nm).

(similarity = 97%; e-value = 0.0) (JX406180).

The 20-gene concatenated phylogeny determined that *R. grylli* from an isopod and *R. isopodorum* are the most related taxa with genome sequence data to '*Candidatus* Aquirickettsiella gammari' (Fig. 6). In Fig. 6, the two isolates from terrestrial isopods group together at 100% bootstrap confidence, and '*Candidatus* Aquirickettsiella gammari' branches below them, with a branch distance of 0.4 units (ML) from *R. grylli* from an isopod and 0.6 units (ML) from *R. isopodorum*. *Diplorickettsia massilisensis* is also closely grouped with these three isolates, at a branch distance of 0.4 units (ML) from '*Candidatus* Aquirickettsiella gammari'.

The phylogenetic tree representing the 16S rRNA gene of many available uncategorised isolates, *Rickettsiella* sp., or other Coxiellaceae, outlines an interesting result whereby '*Candidatus* Aquirickettsiella

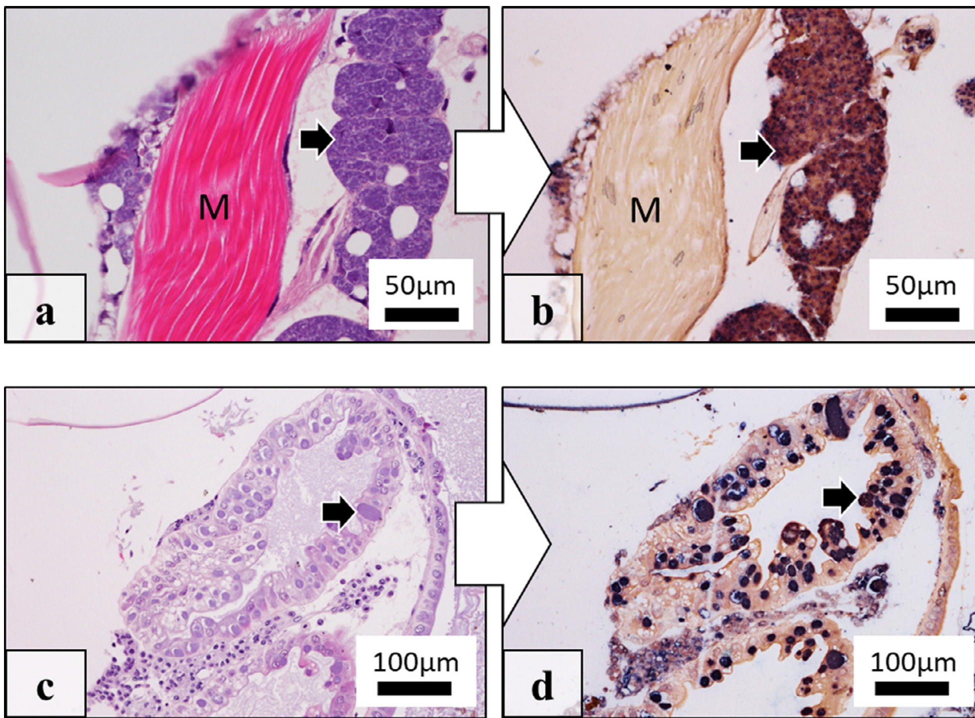

Fig. 4. In-situ hybridisation of bacterial gene probes to histopathologies. a-b) Bacteria in the muscle and haemocytes are detected using an in-situ probe; where a H&E slide (a) with infection (black arrow) is compared to the in-situ slide (b), which has infection stained in blue. Scale = 50 µm. (c, d) Bacteria in the hepatopancreas are detected using the same in-situ probe; where a H&E slide (c) with infection (black arrow) is compared to the in-situ slide (d), which has infection stained in blue. Scale = 100 µm.

gammari' sits outside of the terrestrial *Rickettsiella*, grouping with only aquatic bacterial isolates (Fig. 7). The single gene phylogeny showed a strong 96/80% bootstrap confidence support (NJ/ML) for the separation between the *Rickettsiella* spp. isolated from terrestrial environments/hosts, and those isolated from aquatic environments/hosts (Fig. 7). The 16S phylogeny also determined that *R. isopodorum* and *R. armidillidii* [now thought to be the same species (Kleespies et al., 2014)] branch separately to those *Rickettsiella* sp. that infect insect hosts (62/49% bootstrap confidence) and group together with 100% bootstrap confidence. Additionally, the *R. grylli* isolate (from an isopod) (NZAAQJ02000001) branches just above the *Rickettsiella* isolates from isopods at low-mid bootstrap confidence (51/49%).

One species, *R. viridis*, branches early within the tree, and outside of the *Rickettsiella*, with 100/100% bootstrap confidence. The closest branching species on the tree to *R. viridis* is *Diplorickettsia massiliensis* (0.09 substitutions per site), which sits between *R. viridis*, the *Rickettsiella* and '*Candidatus* Aquirickettsiella'. Whether this suggests that *R. viridis* is a member of the *Diplorickettsia* requires further research.

Based upon the 16S rRNA gene sequence of this novel bacterium and closely related rDNA sequences from NCBI, along with ultrastructural differences (such as the lack of crystalline protein formation within the spherical initial body stage) between the terrestrial insect-infecting *Rickettsiella* and the aquatic crustacean-infecting bacteria described here, we suggest a new candidate genus, '*Candidatus* Aquirickettsiella', to contain this set of aquatic, crustacean-infecting bacteria until the bacteria can be cultured for full genus status, at which time this should be reassessed.

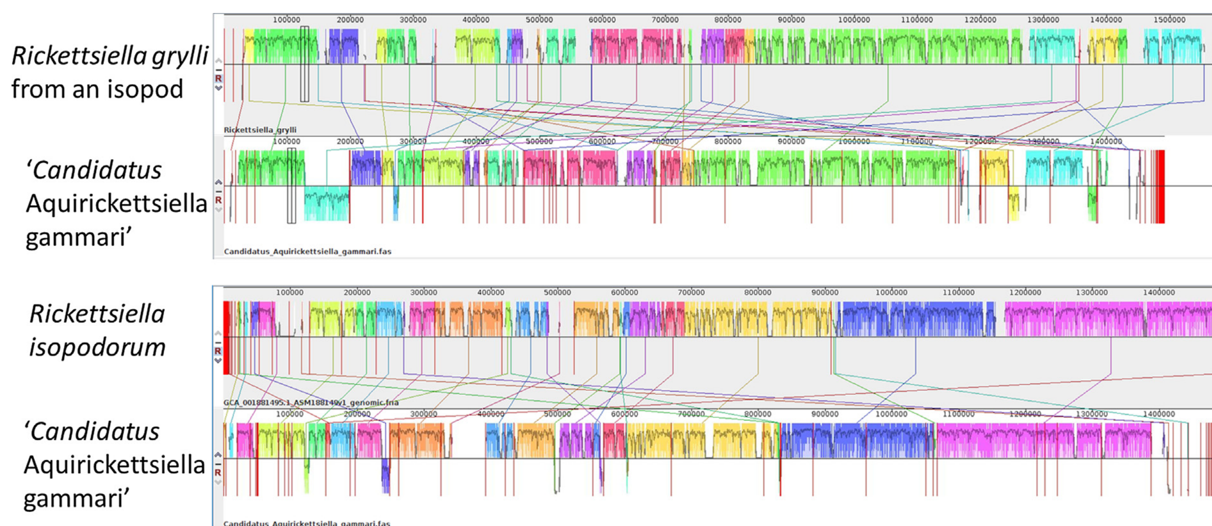

Fig. 5. '*Candidatus* Aquirickettsiella gammari' scaffold comparison to the genome of *Rickettsiella grylli* from an isopod (NZAAQJ02000001) and genome of *Rickettsiella isopodorum* (NZLUKY00000000). These assessments do not determine the actual order of the scaffolds in the true genome of '*Candidatus* Aquirickettsiella gammari' but refers to genomic arrangement comparisons. The colours between the two comparisons in this graph do not correspond.

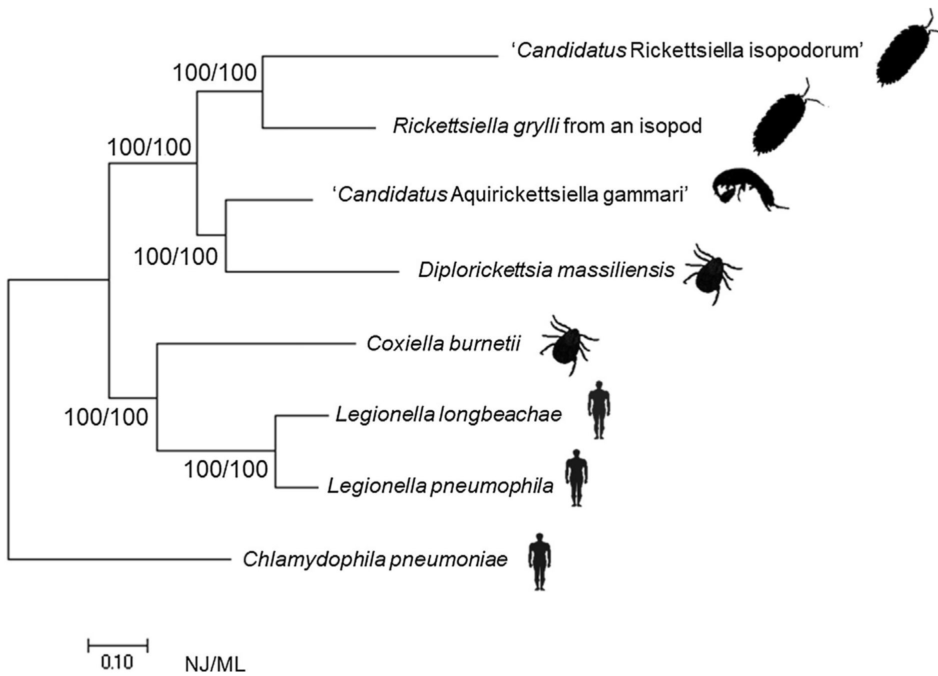

Fig. 6. Phylogenetic placement of '*Candidatus* Aquirickettsiella gammari' using a 20-gene concatenated phylogeny, relative to other related bacterial species from other Crustacea, arachnids and humans, with the available gene complement needed for concatenated sequence analysis. The evolutionary history was inferred by NJ/ML based on the Tamura 3-parameter model. The tree with the highest log likelihood of the ML analysis (-166814.4006) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying NJ and BioNJ algorithms to a matrix of pairwise distances using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The tree is to scale, with branch lengths measured in the number of substitutions per site. There was a total of 20,637 positions in the final dataset. For the NCBI references of the genes used to develop this figure please refer to Supplementary Table 2.

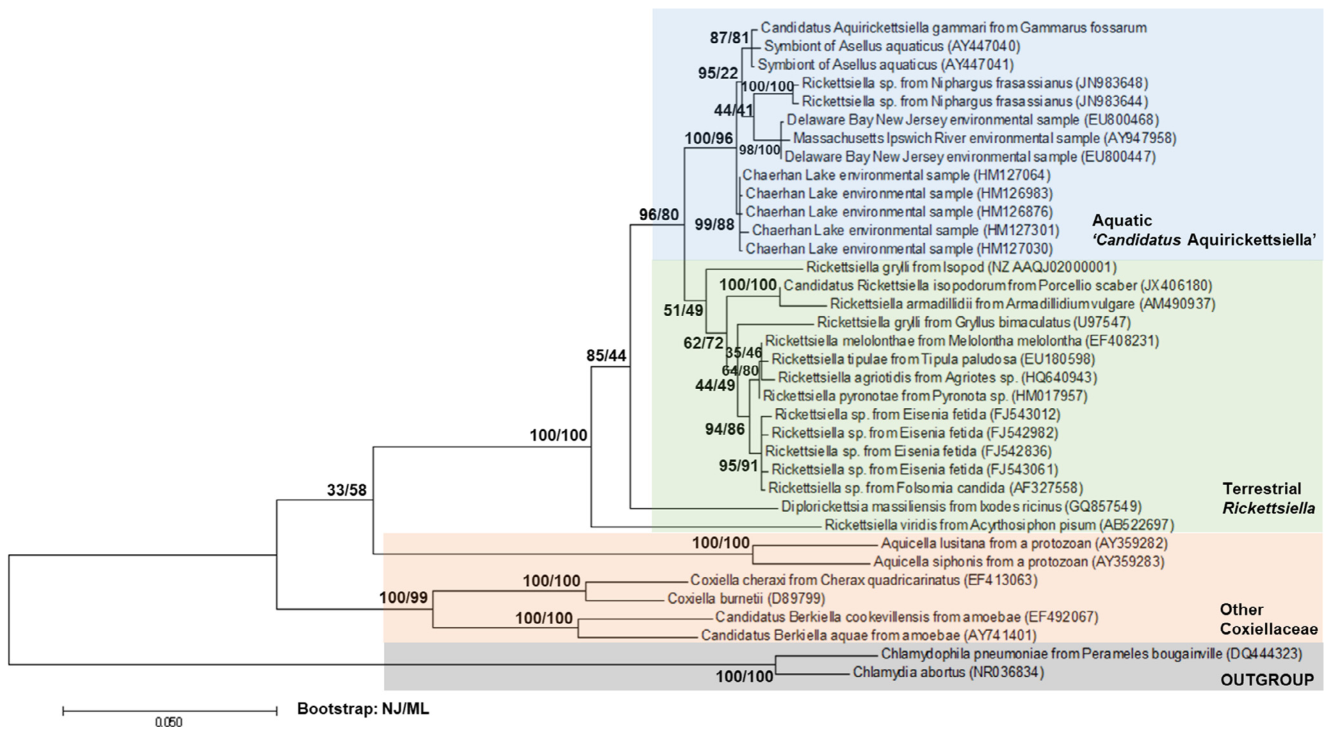

Fig. 7. A phylogenetic tree of the available 16S rRNA gene sequences for several bacterial species, closely and distantly related to '*Candidatus* Aquirickettsiella gammari' (black arrow). The evolutionary history was inferred using an NJ algorithm based on the Jukes and Cantor model, and an ML algorithm based on the Tamura 3-parameter model. The tree shown is from the ML analysis and annotated with the results from both analyses. Both analyses involved 16S rRNA sequences of 36 species. Each genus/family group is indicated with a coloured box, and the outgroup is indicated in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Taxonomic description for candidate genus and species

4.1. Higher taxonomic rankings of 'Candidatus Aquirickettsiella gammari'

Domain: Prokaryota
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Legionellales
Family: Coxiellaceae

'*Candidatus* Aquirickettsiella' Bojko, Dunn, Stebbing, van Aerle, Bacela-Spychalska, Bean, Urrutia and Stentiford, 2018.

Intracellular bacterial organisms, which are pathogenic for crustaceans in aquatic environments. Crystalline inclusions present in insect-infecting *Rickettsiella* are not present in Peracarid-infecting '*Candidatus* Aquirickettsiella'; however, fibrous inclusions are present in those

bacteria infected hepatopancreatic tissues. The bacterium infects the cell cytoplasm of hepatopancreatic epithelia (where morphology can vary), musculature, gill, gonad, nerve and haemocytes, manifesting in late stages as systemic infection. Externally visible pathologies include a white iridescent appearance to infected amphipods, particularly their muscle tissues. The bacterium passes through a four-step development cycle including: the elementary body (smallest developmental stage); an elliptical, condensed sphere stage; division; and a spherical initial body (but not necessarily in that order). All developmental stages take place within a vacuole separating the bacteria from the host cell cytoplasm; however, the elementary body (infective stage) is predicted to be able to survive outside the host cell. Genome sequence data of novel species must show close relatedness through the phylogenetic methods used by this study, and gene conservation relative to the type species.

Type species: '*Candidatus* Aquirickettsiella gammari' Bojko, Dunn, Stebbing, van Aerle, Bacela-Spychalska, Bean, Urrutia and Stentiford, 2018.

This species is intracellular in organs and tissues of the host, *Gammarus fossarum*, including the cells of the hepatopancreas, musculature, connective tissues, nervous system, gonad, gill and, the haemocytes. Heavy infection causes hosts to appear creamy-white, and often iridescent with orange beads running along either side of the pereon. The ultrastructure of the elementary body is composed of an outer membrane measuring 496.73 nm ± 37.56 nm (n = 20) in length, and 176.89 nm ± 36.29 nm in width and is present with an electron dense core and electron lucent lamella. Development includes the elementary body, an elliptical condensed sphere stage, which undergoes division, and includes an initial spherical body stage. Initial spherical body stages do not appear to contain crystalline substances observed in other members of the family, but some fibrous elements are visible in the separate phenotype displayed by the bacterium infecting the hepatopancreatocytes. '*Candidatus* Aquirickettsiella gammari' can be discriminated from other members of the family, and presumably newly discovered members of the genus, by 16S rDNA phylogenies, or construction of concatenated phylogenies based upon the multi-gene sequences as described in this study.

Type host: *Gammarus fossarum* (Gammaridae).

Type locality: Bzura River in Łódź (Łagiewniki) (N51.824829, E19.459828).

Site of infection: The hepatopancreas is proposed as the seat of infection and may precede wider dissemination of the pathogen to cells of the musculature, nerves, gills gonads, connective tissues and, the haemocytes. Etymology: The genus name "*Aquirickettsiella*" is based upon the similarity between this genus and the sister genus *Rickettsiella*, whilst referring to the aquatic habitat and host in which the type species was detected. The specific epithet "*gammari*" refers to the aquatic gammarid host of '*Candidatus* Aquirickettsiella gammari'.

Type material: Histological, TEM and ethanol-fixed material is deposited within the Registry of Aquatic Pathology, Cefas, UK. Data pertaining to the 16S rDNA gene, next generation sequence data and assembled scaffolds for the pathogen and metagenomic dataset generally, is deposited at the NCBI database. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession (s): NMOS00000000/PRJNA392245. The version described in this paper is version NMOS01000000.

5. Discussion

This study describes a novel intracellular bacterial pathogen infecting *G. fossarum* native to continental Europe (Poland), named herein as '*Candidatus* Aquirickettsiella gammari' using histology, ISH, TEM, single-gene and multi-gene phylogenies. '*Candidatus* Aquirickettsiella gammari' is closely related to previously described pathogens of terrestrial arthropods and may be of interest as a biological control agent for invasive gammarid species.

5.1. Taxonomy of '*Candidatus Aquirickettsiella gammari*'

Considering the ultrastructural, histological, genomic and single/multi-gene phylogenies detailed in this study, the aquatic relations of the *Rickettsiella* display some significant differences to terrestrial species. Several insects and some terrestrial isopods have been shown to be infected by members of the genus *Rickettsiella* (Krieg, 1955; Roux et al., 1997; Leclerque and Kleespies, 2008; Leclerque et al., 2011; Kleespies et al., 2011; Leclerque et al., 2012; Tsuchida et al., 2014; Cordaux et al., 2007; Kleespies et al., 2014). Phylogenetic analyses conducted in this study suggest that, within the *Rickettsiella*, a divergence (62/72% bootstrap support) is seen between those species infecting terrestrial crustaceans and those infecting terrestrial insects (Fig. 7). Expanding upon this, a divergence (96/80% bootstrap support) is seen between those bacteria isolated from aquatic hosts/environments relative to those collected from terrestrial hosts/environments (Fig. 7), signifying a likely terrestrial clade (*Rickettsiella*) and an aquatic clade ('*Candidatus* Aquirickettsiella') of this intracellular bacterial group. The concatenated phylogeny suggests that '*Candidatus* Aquirickettsiella gammari' branches closer to *D. massiliensis*; however, '*Candidatus* Aquirickettsiella gammari' is at equal branch distance between the *Diplorickettsiella* and *Rickettsiella* (0.4 Units). This suggests that the true position of this genus in the Coxiellaceae at the phylogenomic level needs to be reconsidered when more species have sequence data to further explore the phylogeny. Despite this, the large branch distance from the *Diplorickettsiella* and *Rickettsiella* suggests that the *Aqirickettsiella* is supported as a novel genus (Fig. 6).

Histology identified this bacterial species to cause gross pathology in the host by infecting the haemocytes, hepatopancreas, muscle sarcolemma, connective tissues, gill, gonad, and nerve tissues. This suggests it is pathogenic to the host, but survival rate when infected is yet to be studied. When bacterial morphology is considered, one primary feature mentioned in the initial genus description of *Rickettsiella* (Philip, 1956) is the inclusion of crystalline protein production within the 'initial body' development stage (see also: Vago et al., 1970; Kleespies et al., 2014). This is apparently missing from those pathogens shown to infect aquatic crustaceans (Federici et al., 1974; Larsson, 1982; This Study). The lack of intracellular crystalline protein formation in the initial body development; the divergence in the 16S rRNA gene between aquatic and terrestrial isolates (Fig. 7); and the branching distance between '*Candidatus* Aquirickettsiella gammari' and other *Rickettsiella* (Fig. 6) provides the basis for an erection of a novel candidate bacterial genus to include the novel bacterium described herein.

As more *Aquirickettsiella* spp. are characterised, such as the two *Rickettsiella*-like bacterial isolates from *Asellus aquaticus* (AY447040/AY447041) (Fig. 7), or when those from *G. pulex* and *C. floridanus* are provided with 16S sequence data, the solidarity of this candidate genus and the phylogenetic analyses should be reassessed.

Currently, no information exists on whether '*Candidatus* Aquirickettsiella gammari' is acquired vertically or horizontally by the host. In addition, because of a lack of crustacean cell culture techniques, it is not possible to axenically culture this bacterial species, although a study assessing this must be formally carried out, and so this genus and species must remain in candidacy until a culture is attempted and successfully carried out.

5.2. Genome composition and annotation for '*Candidatus Aquirickettsiella gammari*'

This study identified 65 contigs associated with '*Candidatus* Aquirickettsiella gammari' from the tissues of *G. fossarum* that show closest similarity to *R. isopodorum* and *R. grylli*, as well some LCBs. Several of the genes isolated from the genomic fragments have homologues that associate to well-characterised pathogens, such as *Legionella* sp. (Edelstein et al., 1999; Albert-Weissenberger et al., 2007). *Legionella* sp. have been used in model systems to identify which genes are

involved in the infection process and several studies like the one by Edelstein et al. (1999) have identified that Type IV secretion systems and conjugal transfer proteins are important for virulence. Such studies are yet to be conducted in bacterial taxa more closely related to '*Candidatus* Aquirickettsiella'; however, parallels can be drawn for certain homologues in both '*Candidatus* Aquirickettsiella gammari' and *Rickettsiella* sp. isolated from isopods. Both species include Dot-like genes, Icm-like genes and conjugal transfer proteins (Tra) that are homologous to those found in *Legionella*. Only '*Candidatus* Aquirickettsiella gammari' encodes Vir-like proteins homologous to those found in *Legionella*, *Tatlockia* and *Diplorickettsia*. The presence of several genes associating to the Type IV secretion system in the genome of '*Candidatus* Aquirickettsiella gammari' suggests it has the capability to introduce genetic material to its hosts cells, a process which may be similar to the well-characterised pathway used by *Agrobacterium tumefaciens* to engineer its hosts cell cycle to suit its own development needs (Wood et al., 2001; Tzfira and Citovsky, 2006). Plants infected with the wild-type, pathogenic, *A. tumefaciens* produce localised cellular growths to form a "gall" (Wood et al., 2001; Tzfira and Citovsky, 2006). For '*Candidatus* Aquirickettsiella gammari', the histopathology data revealed several infected tissue types, all of which were undergoing relatively large levels of hypertrophy; in particular, the infected haemocytes and connective tissues had adhered to one another forming large masses in the circulatory system of the host (Fig. 1a). Although speculation at this point, this species and the systems encoded by its genome may provide a useful insight for future studies exploring the introduction of genetic material to crustacean tissues via bacterial horizontal methods.

5.3. Why characterise the pathogens of native amphipod hosts?

Most taxa are evolutionarily adapted to survive in particular settings, but when transferred to new surroundings those taxa may either thrive and become invasive or perish and be removed from the community. Amphipods are renowned for their capability to spread and colonise water systems, and several studies have assessed their hardiness (Bruijs et al., 2001), behaviour (Dick et al., 2002) and ability to spread (Bacela-Spychalska, 2016); even suggesting some are "perfect invaders" (Rewicz et al., 2014). With impending invasion comes the possibility to co-introduce disease (Dunn and Hatcher, 2015), or escape from disease, allowing the host to become fitter and more competitive in its new territory (Colautti et al., 2004). Invasions threaten biological diversity (Lambertini et al., 2011) and finding natural enemies that may control invasive species is one possible mode to negate an invader's impacts. A recent study by Bojko et al. (2018) has identified that the presence of pathogens co-introduced alongside an invasive amphipod host can both control invasive amphipod characteristics, but also threaten native species that are susceptible to infection, signifying the importance of understanding pathogens before using them as control agents in invasive amphipod research.

By screening an amphipod population from its native environment, it is possible to observe an overview of the naturally associated symbionts before enemy release has taken place. The identification of '*Candidatus* Aquirickettsiella gammari' provides an example of a novel organism similar to those selected for biological control in the past (McNeill et al., 2014; Lacey et al., 2015). This novel pathogen could possibly be adapted into a control agent, but not without firstly conducting further studies upon the effects of the pathogen on the survival of the host, and the pathogens host range. Such studies would relate to the development of biocontrol agents for agricultural settings (Lacey et al., 2015). Such studies would also assess potential risk for this pathogen transfer to native fauna and determine whether it could cause damage to other populations that may co-occur with invasive *G. fossarum* (Blackman et al., 2017).

'*Candidatus* Aquirickettsiella gammari' is the first characterised intracellular bacterial species from an amphipod, and this novel genus likely includes the bacteria identified from *C. floridanus* (Federici et al., 1974), *G. pulex* (Larsson, 1982) and possibly the intracellular bacterial pathogen from the hepatopancreas of non-native *G. roeselii* in Poland (Bojko et al., 2017). This new discovery suggests that the native environments of other invasive amphipods that require control, such as *D. villosus* and *Pontogammarus robustoides*, may hold similar microbial agents that could benefit their biological control.

When invaders co-occur with native gammaridean fauna, including *G. fossarum* inhabiting the lowland rivers of Central Europe, these invasive species may face new pathogens, such as the one described in our study, which could be contracted and may also play a role to regulate their populations. When host range data for '*Candidatus* Aquirickettsiella gammari' is researched, infection trials with high impact invasive amphipods would determine the transmissibility of the pathogen to high profile invaders and could determine if the agent could transmit to, and control, invaders (*D. villosus*, *D. haemobaphes*, *Echinogammarus tirchiatus* and *P. robustoides*) in native Polish freshwater environments.

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JB, AMD, PDS, KBS and GDS designed the original study. JB and KBS collected and preserved the tissues. JB conducted the histology, transmission electron microscopy and PCRs. AU conducted the in-situ hybridisation. JB and TPB prepared the infected host material for Illumina MiSeq. JB and RvA conducted the bioinformatic analysis of the MiSeq data. All authors contributed to the final manuscript. The authors declare that they have no conflicts of interest.

No ethical statement was obtained prior to conducting the sampling work due to the use of invertebrate species (amphipods). Upon collection, animals were either housed before euthanisation (through cooling) to allow dissection or were fixed onsite for histological analysis.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.07.010.

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