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‘*Candidatus* Mellornella promiscua’ n. gen. n. sp. (Alphaproteobacteria: Rickettsiales: Anaplasmataceae): An intracytoplasmic, hepatopancreatic, pathogen of the flatback mud crab, *Eurypanopeus depressus*

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ABSTRACT

Bacterial pathogens are a long-standing threat to the longevity and survival of crustacean hosts. Their presence and continuing emergence require close monitoring to understand their impact on fished, cultured, and wild crustacean populations. We describe a new bacterial pathogen belonging to the Anaplasmataceae family (Alphaproteobacteria: Rickettsiales), providing pathological, ultrastructural, phylogenetic, and genomic evidence to determine a candidate genus and species (‘*Candidatus* Mellornella promiscua’). This bacterium was found to infect the mud crab, *Eurypanopeus depressus,* on the North Carolina coastline (USA) at a prevalence of 10.8%.

‘*Candidatus* Mellornella promiscua’ was often observed in co-infection with the rhizocephalan barnacle, *Loxothylacus panopaei*. The bacterium was only found in the hepatopancreas of the mud crab host, causing cytoplasmic hypertrophy, tubule necrosis, large plaques within the cytoplasm of the host cell, and an abundance of sex-pili. The circular genome of the bacterium is 1,013,119 bp and encodes 939 genes in total. Phylogenetically, the new bacterium branches within the Anaplasmataceae. The genome is dissimilar from other described bacteria, with 16S gene similarity observed at a maximum of 85.3% to a *Wolbachia* endosymbiont.

We explore this novel bacterial pathogen using genomic, phylogenetic, ultrastructural, and pathological methods, discussing these results in light of current bacterial taxonomy, similarity to other bacterial pathogens, and the potential impact upon the surrounding disease ecology of the host and benthic ecosystem.

1. Introduction

Bacterial pathogens of crustacean hosts are taxonomically diverse (Wang, 2011). Bacterial pathogens like *Vibrio parahaemolyticus* the causative agent behind acute hepatopancreatic necrosis diseases (AHPND), and ‘*Candidatus* Hepatobacter penaei’ the cause of necrotizing hepatopancreatitis (NHP) have been implicated in losses of aquacultured Penaeid and Portunid shellfish stocks (Sudheesh and Xu, 2001; Wang, 2011; Nunan et al. 2013; Leobert et al. 2015; Santos et al. 2020). The emergence of pathogenic bacteria in crustacean aquaculture is relatively common and can be costly: the cost of AHPND between 2010 and 2017 was estimated at ~US$ 11.5 billion, including export losses (Shinn et al. 2018). Such economic impacts of emerging bacterial pathogens reflect the great importance of exploring their ecological and evolutionary origins in wild hosts, which could serve as reservoirs for pathogenic bacterial infection in commercially valuable species.

Major pathogenic prokaryotic groups include the intracellular proteobacteria of crustacean hosts, which belong to multiple systematic orders (e.g. Rickettsiales, Mycoplasmatales, Legionellales, Chlamydiales) (Tan and Owens, 2000; Wang et al. 2004; Wang, 2011; Bojko et al. 2018). The Rickettsiales (Class: Alphaproteobacteria) are a common group reported by crustacean pathologists, historically using morphological data alone. Observations include infections in *Carcinus mediterraneus* (Bonami and Pappalardo, 1980), *Paralithodes platypus* (Johnson, 1984), *Callinectes sapidus* (Messick, 1998), and *Lithodes aequispina* (Meyers and Shorts, 1990). The examples above are represented by morphological and/or pathological data, but without genetic confirmation of their phylogeny (Wang, 2011; Leyva, 2018). Genetic

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data can help to untangle taxonomic conundrums, providing detail on evolutionary relationships and relatedness.

The Rickettsiales includes three genetically distinct families: the Rickettsiaceae, the Anaplasmataceae and the '*Candidatus* Midichloriaceae' (Szokoli et al. 2016). Of interest to this study is the family Anaplasmataceae, which includes the genera: *Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, '*Candidatus* Neoanaplasma', '*Candidatus* Neoehrlichia' and *Wolbachia* (Szokoli et al. 2016; Chegeni and Tavakoli, 2018). Members of the Anaplasmataceae use a diverse array of hosts including multiple vertebrates (including humans), arthropods, and protists (Szokoli et al. 2016). Crustacean hosts of Anaplasmataceae include the Isopoda and Amphipoda, which are parasitised with *Wolbachia* that can induce host sex alteration; a process that facilitates vertical transmission of the pathogen but can harm host reproduction (Cordaux et al. 2001; Werren et al. 2008). To date no Anaplasmataceae have been formally identified in crabs (Decapoda: Brachyura), including commercially- and ecologically- valuable portunids (swimming crabs) and panoepids (mud crabs). Within the Panopeidae, specifically, no bacterial pathogens have been reported to our knowledge, as reflected by recent reviews of mud crab symbionts (Moore et al., 2020; Bojko et al. 2021).

This study identifies a novel intracytoplasmic, hepatopancreatic, bacterial pathogen ('*Candidatus* Mellornella promiscua' n. gen. n. sp.) from the flatback mud crab, *Eurypanopeus depressus*, a common and abundant panopeid mud crab native to estuaries of the western Atlantic Ocean and the Gulf of Mexico (Williams, 1984). The novel bacterium was identified using genome comparison techniques, phylogenetics, ultrastructure, intracellular lifecycle, and pathogenic effects. The implications of this new bacterial pathogen are discussed in the context of host-pathogen interactions, relatedness to other pathogenic bacteria, systematics, estuarine ecology, and how this discovery outlines a new lineage of crustacean-infecting bacteria.

2. Materials and methods

2.1. Sampling and dissection

In December 2018, *E. depressus* (n = 65) were collected from Hoop Pole Creek, Bogue Sound, North Carolina (34°42′25.06″N; 76°45′6.84″W) by hand and brought back to the Blakeslee Laboratory (East Carolina University) and kept in separate, fixed-grid, plastic parts boxes (5 cm^3) for 2 days prior to dissection. Individuals were checked morphologically to confirm the host species, sex, and size (carapace width). Animals above 9 mm in carapace width were fully dissected, taking muscle tissue, hepatopancreas and gill for molecular diagnostics (96% ethanol), electron microscopy (2.5% glutaraldehyde in 0.1% sodium cacodylate buffer) and histology (fixation in Davidson's saltwater fixative). These tissues are typically the regions where parasites/pathogens have been found to concentrate based on past examinations of these crabs (Bojko et al. 2021). Animals below 9 mm in size were halved, with one half fixed in 96% ethanol for molecular diagnostics and the second half fixed in Davidson's saltwater fixative for histological preparation. Any visible infection (i.e., the presence of an externa—the parasite's larval sac—on the crab's abdomen) by parasitic barnacles (Rhizocephala) were noted.

2.2. Histopathology

Davidson's fixed tissues were first decalcified (hydrochloric acid and EDTA solution) before being placed into 70% ethanol. Samples were then put through a dehydration series into 100% ethanol, then into a xylene substitute (Slide Brite, Newcommer Supply) before four rounds of wax infiltration using an automated tissue processor (Miles Sakura Tissue-Tek vip 2000). Paraffin (PureAffin X, Newcommer Supply) infiltrated tissues were embedded into blocks (Tanner Scientific TH1550 Embedding Console System) and trimmed to expose the tissue before taking two sections at 3–4 μm on a Leica RM2520 microtome and mounting onto glass slides. The slides were re-hydrated and stained using haematoxylin and alcoholic eosin, then cover-slipped. The slides were read on a Leica microscope and imaged using an integrated Leica camera.

2.3. Transmission electron microscopy (TEM)

To investigate the bacterial ultrastructure, tissues fixed in 2.5% glutaraldehyde in 0.1% sodium cacodylate buffer were processed through two buffer changes and heavy metal stained using osmium tetroxide (OsO_4). Stained tissues were processed through an ethanol dehydration and then infiltrated with LR White resin using an ethanol: resin infiltration series. The tissues were baked into a resin block and trimmed to expose the tissue using a glass knife. Semi-thin sections were taken at 1 μm to identify the section of interest. The block was then trimmed using a diamond knife to produce ultrathin sections (~45 nm), which were stained using lead citrate and uranyl acetate and viewed on a FEI G2 F20 transmission electron microscope (TEM) (200 kV) (Tecnai).

2.4. Next generation sequencing and bacterial genome completion

Ethanol-preserved hepatopancreas from the same individual used for TEM underwent DNA extraction using a Zymo extraction kit, as per manufacturer instructions. The extract was submitted to Novogene (California) and checked for DNA degradation (1% agarose gel), DNA purity (OD260/OD280, OD260/OD230) (NanoPhotometer® spectrophotometer: IMPLEN, CA, USA), and DNA concentration (Qbit® 2.0 Flurometer: Life Technologies, CA, USA).

A total amount of 1 μg of DNA was used to prepare the library. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations. The DNA sample was fragmented by sonication to ~150 bp, which were end-polished and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. PCR products were purified (AMPure XP system) and the library was analysed for size distribution using an Agilent2100 Bioanalyzer and quantified using real-time PCR. The clustering of the index-coded sample was performed on a cBot Cluster Generation System according to manufacturer instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated.

Sequence data were collected by Novogene and delivered electronically to the University of Florida for bioinformatic analysis. The data were first trimmed using Trimmomatic v0.36 (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (Bolger et al. 2014). Paired end reads were assembled in combination with unpaired data using SPAdes v3.13.0 (Bankevich et al. 2012), with additional parameters (forward-reverse reads; 28 threads; 500 Gb RAM; no read cut-off; 64 phred-offset) and k-mer lengths of 21, 33, 55, 77, 99.

The assembled data resulted in 523,047 contiguous DNA sequences (>500 bp) (N50: 1961, N75: 1165, L50: 117245, L75: 251727), 8 of which were associated with the bacterial genome of the novel species, using blast similarity searches in NCBI (blastn/blastp/blastx). Manual extension using the sequence data successfully connected some bacterial contigs where others required PCR confirmation. Five remaining genomic gaps were completed using PCR, with conditions and primers for each described in Table 1. PCR amplicons were purified using ExoSAP-IT (ThermoFisher Scientific), and purified amplicons were then Sanger sequenced in the forward direction by Macrogen USA (Rockville, Maryland).

The final genome (1,013,119 bp) was annotated for open reading frames using GeneMarkS (Besemer et al. 2001), the prokaryotic genome annotation pipeline, and GeneMarkS-2 + provided by NCBI and determined to encode 939 total genes. The genome is stored in NCBI under

Table 1
Thermocycler conditions and the primer designs for PCRs conducted to complete the bacterial genome and close those contigs identified from the next generation sequencing efforts. The initial denature temperature was 94 °C (4 min) and the final elongation step was 72 °C (7 min) for all PCRs.

Target	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Thermocycler conditions (°C)	Cycles
Area 1	CTGGAGACTCCTCATCATTATGCT	CAGCCTCAATCTGAAATGCCA	94(1 min)/57(1 min)/72(1 min)	40
Area 2	GCATCTCGTCCTAACCTTCT	CGAAAGGTCGTCAGATTCCA	94(1 min)/55(1 min)/72(1 min)	40
Area 3	CTTGTGCACTAGAATGT	CGAACATGTCATGAATGT	94(1 min)/46(1 min)/72(1 min)	40
Area 4	CTCTGGTCTATCCGGTATGCCT	CTGGCCTTTCATCAAAGCAAT	94(1 min)/55(1 min)/72(1 min)	40
Area 5	CACATGGCCTATCGT	CTTCAATGTTTGGATATGTCAAT	94(1 min)/50.5(1 min)/72(1 min)	40

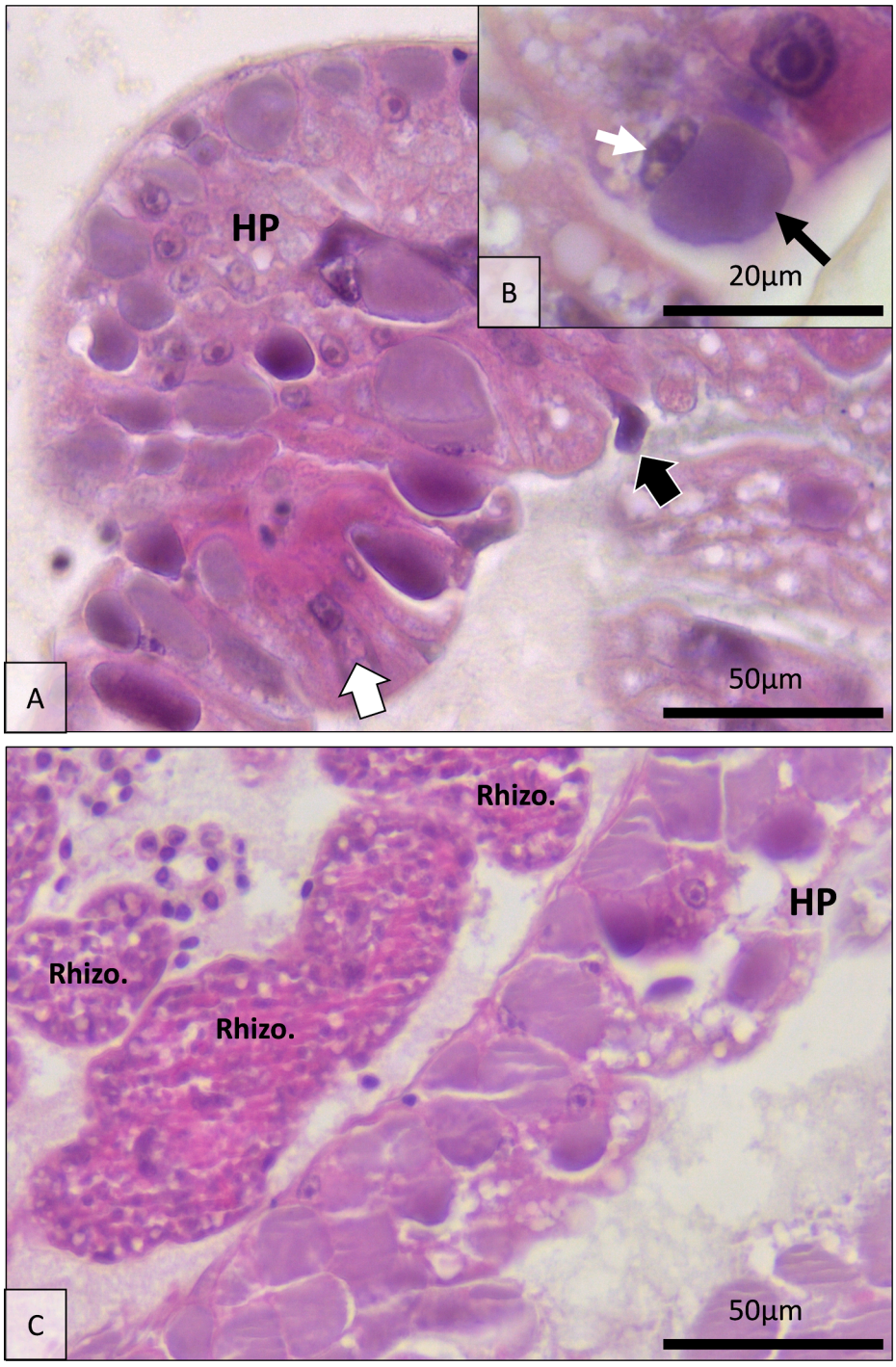

Fig. 1. Histological sections of the mud crab hepatopancreas. A) A single hepatopancreatic tubule exhibiting infection by '*Candidatus* Mellornella promiscua'. An uninfected cell is highlighted using a white arrow. Ejection of a bacterial plaque into the hepatopancreatic lumen is highlighted by a black arrow. B) A growing vacuole containing the developing bacterial infection is identified using a black arrow. The nucleus of the host cell is identified using a white arrow. C) Multiple infected hosts also presented infection with a Rhizocephalan barnacle (Rhizo.). The infected hepatopancreatic tubule (HP) is present alongside the rhizocephalan tendril. The rhizocephalan does not appear to exhibit infection by the bacteria.

accession: CP048228. Genome coverage and continuity was checked using CLC genomics workbench v.11 and Circa was used to develop a graphical representation of the genome (omgenomics.com/circa/).

2.5. Phylogenetic and other nucleotide/protein comparisons

The complete 16S gene was isolated from the genome of the novel bacterium and aligned with the 16S sequences from 29 other members from across the Rickettsiales. Alignment of the sequence data was conducted using MAFFT via the CIPRES gateway (Katoh and Toh, 2010) and then submitted to IQtree (Minh et al. 2020). The final alignment was over 1203 columns and the maximum-likelihood tree was based on the following evolutionary model according to BIC: TVMe + I + G4, using 1000 bootstrap replicates. The resulting tree was downloaded and annotated using FigTree v1.4.3.

OrthoFinder v2.5.2. (Emms and Kelly, 2019) was used to concatenate protein sequence data from 13 Rickettsiales members, including our new isolate. The sequences were isolated using Diamond search program and MAFFT alignment before tree inference via dendroblast, within OrthoFinder. MCL inflation parameter was set to 1.5 (default). The resulting tree was inferred by STAG (Emms and Kelly, 2019) and mid-point rooted in FigTree v.1.4.3.

BLASTn, BLASTx and BLASTp (NCBI) were used to conduct and predict gene/protein function and compare similarity to other bacterial

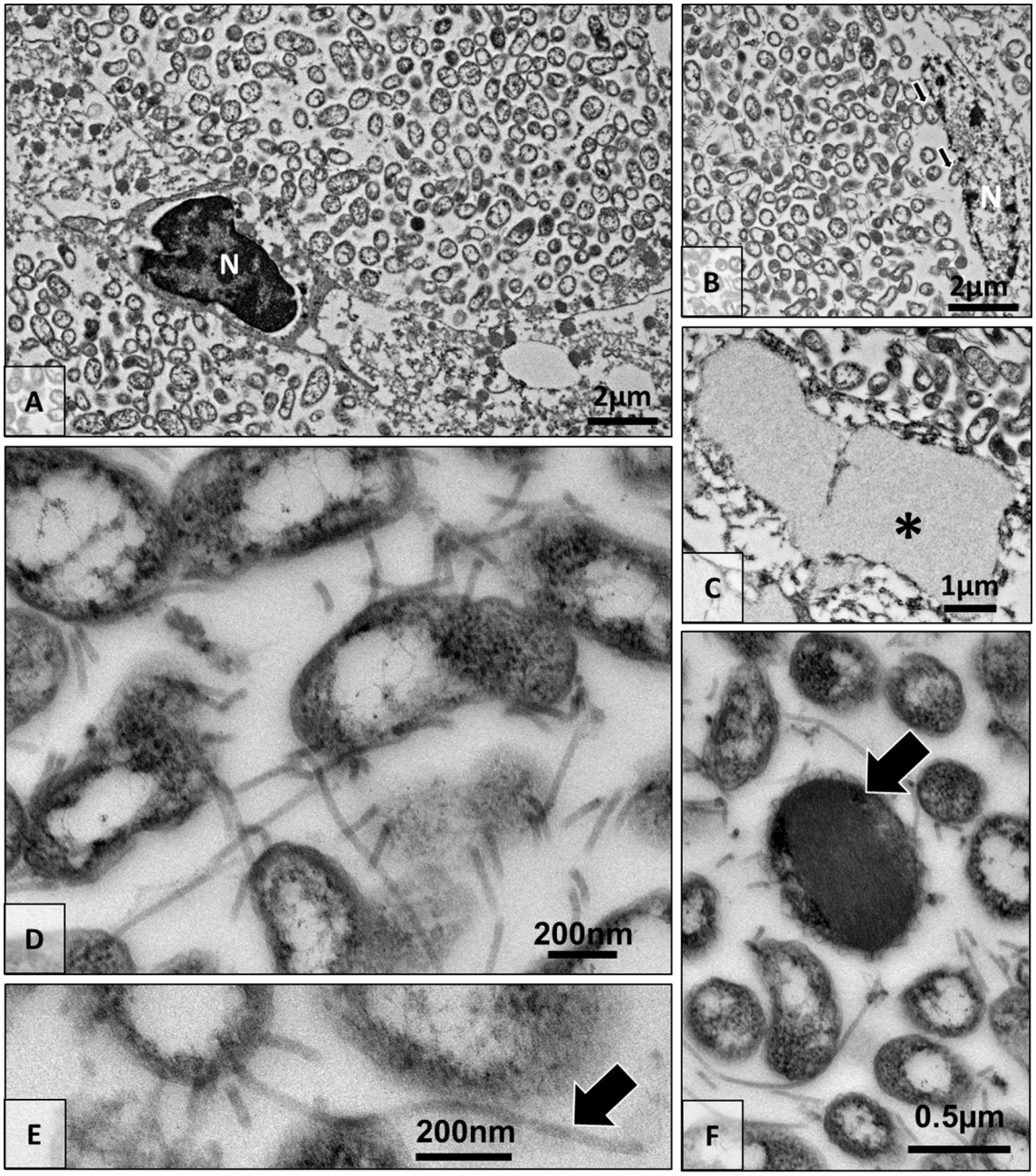

Fig. 2. Transmission electron micrographs of the intracellular bacterial infection caused by '*Candidatus* Mellornella promiscua'. A) A high magnification image of multiple adjoining host cells with bacterial endosymbionts. A host nucleus (N) is in section. B) In some advanced infections the bacteria appear to be present in direct contact with the host cytoplasm and are in close contact (black arrows) with the host nucleus (N). C) A build-up of an undetermined chemical (*) was present in the cytoplasm of some host cells and was surrounded by the bacteria. D-E) The bacteria were commonly seen with pili-like structures connecting them with other bacterial endosymbionts within the vacuole (black arrow). F) Some of the bacterial cells were observed to have inclusion bodies, which were fibrous, and protein-like, in their cytoplasm (black arrow). These cells were slightly larger than those cells without the inclusion.

isolates. AntiSMASH v6.0.1. (Blin, 2021) was used to look for putative secondary metabolite production.

2.6. Statistical analyses

Data attained from the histology slides were recorded in a binomial fashion, representing the presence (1) or absence (0) of each parasite noted during the survey. A Fisher's exact test was performed in GraphPad (https://www.graphpad.com/quickcalcs/contingency2/) to examine whether bacteria were more likely to occur in the presence of rhizocephalan barnacles (one-tailed test).

3. Results

3.1. Bacterial prevalence and co-infection

Eurypanopeus depressus were histologically identified to harbour a basophilic, intracytoplasmic bacterial pathogen in all cell types of the hepatopancreas (Fig. 1a-c). Basophilic inclusions are noted to exhibit different affinities for the stain, some a deeper purple than others. From a total sample size of 65 individuals, 7 were found histologically with the bacterial pathogen (10.8% prevalence). The histopathology of the infection was represented by basophilic intracytoplasmic inclusions that reached the periphery of the cell and caused cytoplasmic hypertrophy

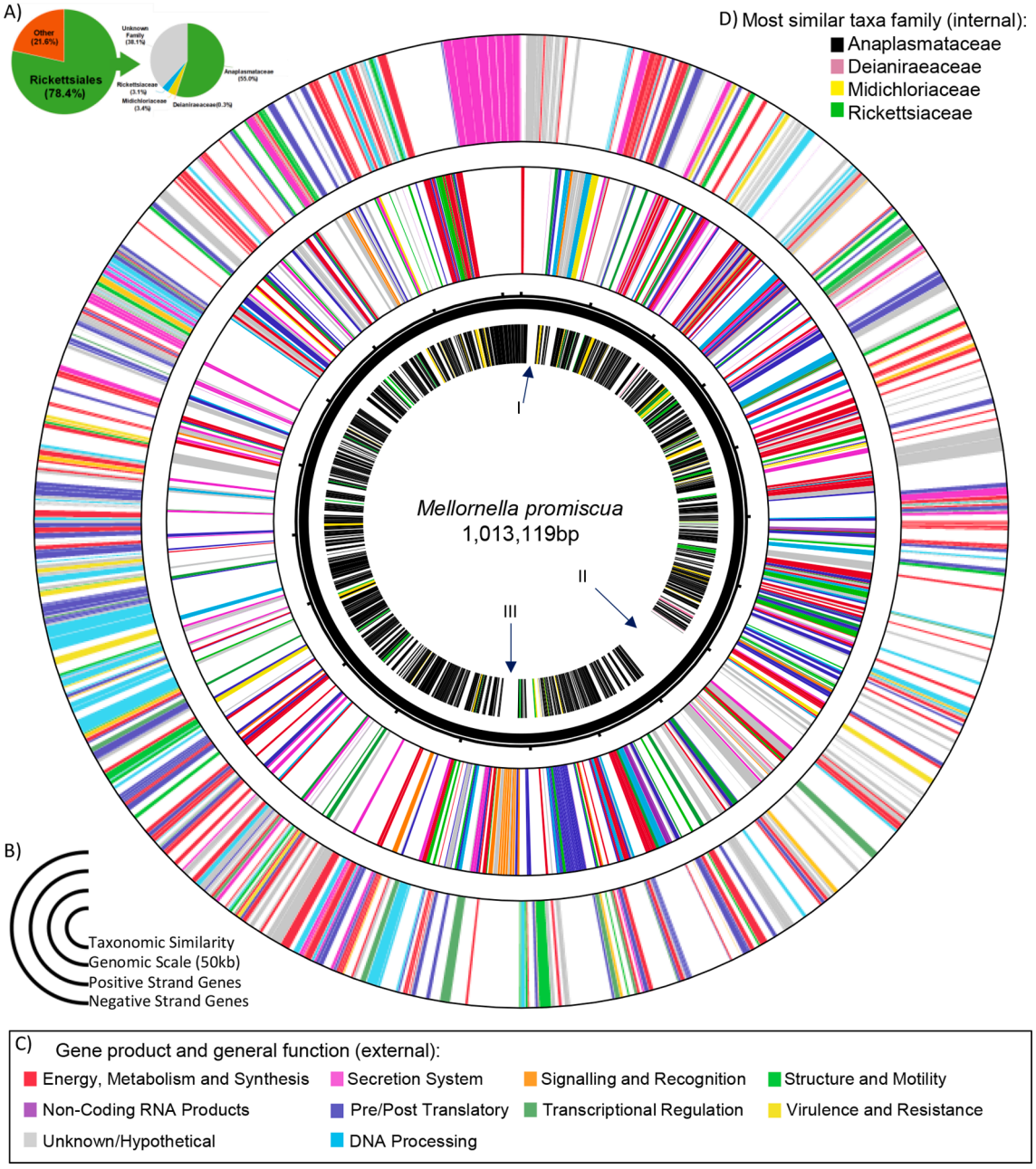

Fig. 3. A graphical representation of the '*Candidatus* Mellornella promiscua' genome (1,013,119 bp), including gene annotation and relative taxonomic similarity. A) A pie chart of the total number of protein coding genes (PCGs) reflecting their protein similarity to the Rickettsiales and internal families. B) A key for the layout of the central genome graphic. C) A key to recognise the function of the annotated genes. D) A key to recognise the 'taxonomic similarity' of each gene to the most related Rickettsiales family. The roman numerals internally refer to specific areas and gene clusters that show little resemblance to other Rickettsiales and may have alternative origins (Table 2). Further information, including a detailed breakdown of the proteins and predicted function, relating to this Fig. can be found by accessing the NCBI accession (CP048228). The Fig. was developed using Circa (omgenomics.com/circa/).

(Fig. 1a-b). Observation of the parasitic rhizocephalan barnacle, *Loxothylacus panopaei*, during dissection and histological screening revealed that 25 of the individuals we sampled had a Rhizocephalan infection, and six of the seven bacterially infected animals had at least one rhizocephalan parasite (Fig. 1c). There was a significant association between bacteria and barnacles, (Fisher's exact test, $p = 0.011$), indicating that crabs were more likely to have bacteria if they also had barnacles than would be expected by chance. Additional observations of bacterially infected individuals via histological section included ectocommensal gill/cuticle ciliates in 2/7 animals, and 1/7 with a nematode infection.

3.2. Bacterial ultrastructure and development

Transmission electron micrographs of the intracytoplasmic inclusions identified multiple bacterial vacuoles expanding in the cell cytoplasm (Fig. 2a). Each bacterium measured 0.591 ± 0.075 μm (mean standard deviation; n = 20) in width and 0.731 ± 0.088 μm (n = 20) in length. The bacteria were typically present within a vacuolar membrane, but in more advanced infections with higher numbers of bacteria, they were no longer held within the vacuolar membrane and were in direct contact with the host cell cytoplasm (Fig. 2b). In some instances, the bacteria were in direct contact with the nucleus of the host cell (Fig. 2b). In some vacuoles, an unknown chemical build-up was observed and commonly surrounded by the resident bacteria (Fig. 2c). Most bacteria were present with pili-like structures extending from multiple locations around the bacterial cell (Fig. 2d-e). Most commonly, the bacteria contained vacuoles, but some were filled with a fibrous inclusion (Fig. 2f), growing to 0.872 ± 0.004 μm (n = 4).

3.3. Genome composition, function, and genetic relatedness

The circular genome of '*Ca.* Mellornella promiscua' was 1,013,119 bp in size and encoded 899 predicted protein coding genes, 1 pseudogene, 3 ncRNA genes, 3 rRNA genes, and 33 tRNA genes. (Fig. 3). The PCGs (GeneMarkS) encode a variety of functional proteins, including: energy, metabolism and synthesis (n = 216); DNA processing (n = 82); secretion (n = 65); translation (n = 181); signalling and recognition (n = 34); transcriptional regulation (n = 39); structure and motility (n = 64); virulence and resistance (n = 17); and some of unknown/hypothetical function (n = 201) at the time of analysis (Fig. 3). The genome appears to include three major regions that encode PCGs with low similarity to organisms within the Rickettsiales. These three regions are located around the genome: I (5255 bp – 16,942), II (351,416 bp – 381,560 bp) and III (509,795 bp – 522,550 bp) (Table 2).

'Region I' includes 12 protein coding genes, which broadly associate to a mix of Alphaproteobacteria (including *Zymomonas mobilis*), Gammaproteobacteria, and *Cetobacterium* sp., and function as a mix of predicted structural or metabolic proteins. Region II is the largest region, spanning ~30kbp, and encodes two primary protein groups, one linked to molybdenum biosynthesis and one to nitrogen processing. Molybdenum biosynthesis genes show the greatest similarity to the Rhizobiales (Alphaproteobacteria), and the nitrogen processing genes show conservation, including both high similarity and order, to a member of the Pelagibacteriales (Alphaproteobacteria) (Table 2). Region III PCGs are predicted to have a signalling function; however, the majority show little similarity to other known bacterial isolates (Table 2).

Many of the annotated genes have no recognisable similarity to the aforementioned groups, or show similarity to isolates and species outside of these groups (354 PCGs). Others show greatest relatedness to members of the Anaplasmataceae (n = 510), Deianiraeaceae (n = 3), Midichloriaceae (n = 32) and Rickettsiaceae (n = 29) (Fig. 3). The most related genus to '*Ca.* Mellornella promiscua' is *Neorickettsia*, which is supported by maximum-likelihood phylogenetic analysis using the 16S rRNA gene (Fig. 4) as well as a multi-orthologue group STAG tree (Fig. 5). BLASTn comparison of the 16S gene to other bacterial isolates stored in NCBI revealed that the closest known isolate to '*Ca.* Mellornella promiscua' is an uncultured bacterial species (85.69% similarity; 93% coverage; e-value: 0.0; accession: JN538034) from a hypersaline pond in Guerrero Negro, Mexico. The closest isolates that have been systematically characterised, based on 16S comparison, are a *Wolbachia* endosymbiont of *Torotrogla cardueli* (85.30% similarity; 93% coverage; e-value: 0.0; accession: KP114101) and '*Ca.* Neoehrlichia mikurensis' (85.04% similarity; 94% coverage; e-value: 0.0; accession: AB196304).

Virulence genes in '*Ca.* Mellornella promiscua' include multiple virulence factors, drug resistance genes, and phage-associated proteins. Genes 27, 92, 330, and 671 all relate to penicillin or other antimicrobial drug resistance. Virulence factors are present, including genes 213–215 (VirB2) and 881 (YihY). Finally, genes 69 (phage portal protein), 332 (phage terminase) and 737 (phage major capsid protein) relate to endogenous viral integration or viral susceptibility genes. Gene 69 shows closest protein relatedness to a *Wolbachia* symbiont of *Pentalonia nigronervosa* (46.17% similarity; 91% coverage; e-value: 2e-109; WP188153605). Using Struct2Net (Singh et al. 2010), the major capsid protein (QEJ80817) and minor capsid protein (QEJ80820) of the *Jodiemicrovirus 1* (Bojko et al. 2019), are unlikely to interact with the phage portal protein of '*Ca.* Mellornella promiscua'. Gene 332 shows greatest similarity with a *Wolbachia* sp. (43.23% similarity; 99% coverage; e-value: 9e-124; WP012481978); however, comparing this to viral taxa (taxid: 10239) in BLASTp suggests it may be derived from a Mu-like cryoconite phage (unclassified virus group) (32.75% similarity; 97% coverage; e-value: 4e-74; QMV49911) or a member of the *Caudovirales* (32.33% similarity; 94% coverage; e-value: 7e-73; CAB4214482). Similarly, gene 737 of the GeneMarkS annotation, an integrated major capsid protein gene, shows greatest resemblance to an EVE of *Ehrlichia chaffeensis* (34.63% similarity; 97% coverage; e-value: 4e-56; WP006009762); however, greatest viral relatedness is to the *Caudovirales* (27.40% similarity; 96% coverage; e-value: 2e-35; YP355412).

Finally, '*Ca.* Mellornella promiscua' appears to have multiple predicted semi-conserved biosynthetic secondary metabolite clusters using the 'cluster blast' and 'MIBiG comparison' option of antiSMASH (v6.0.1). These are present in the 330,661 bp-351,056 bp (R1), 665,102 bp-686,181 bp (R2) and 898,450 bp-919,406 bp (*R*3) regions. The R1 region includes two genes with similarity to a fatty acid biosynthesis pathway located on the genome of a *Salaquimonas pukyongi* isolate (NZ_CP019044). The two genes include a transport-related gene and a biosynthetic gene. The likely resulting polyketide product (similarity score: 0.17/0.49, species: *Streptomyces arenae*) may resemble naphthocyclinone (reference: BGC0000248).

Region R2 contains four genes predicted to function as a saccharide biosynthesis pathway, most related to a gene cluster found in the genome of *Neisseria zoodegmatis* (NZ_LT906434). MIBiG analysis suggests the resulting product may be like moenomycin, produced by *Streptomyces viridosporus* (similarity score: 0.16/0.47) (reference: BGC0000805). The final cluster located in *R*3 of the bacterial genome includes four genes related to a second fatty acid biosynthesis pathway found on the genome of a *Methylobacterium* sp. (NZ_CP029551). The likely resulting polyketide product (similarity score: 0.15/0.45, species: *Streptomyces* sp.) may resemble viguiepinol (reference: BGC0000286).

4. Taxonomic description

4.1. Higher taxonomy

Domain: Prokaryota
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rickettsiales
Family: Anaplasmataceae

Table 2
Three genomic regions that display gene clusters with greater similarity to taxa outside of the Rickettsiales. These regions may include highly specific genes to the '*Ca.* Mellornella' genus or may have been acquired from other taxonomically separate groups through horizontal transfer.

Genomic Segment	Protein coding gene [−/+ strand, region(bp)]	BLASTP Similarity			NCBI hit and protein function		
		Similarity (%)	Coverage (%)	e-value	taxa	gene	accession
I (5255 bp – 16942 bp)	Gene 3 (−, 5255–5989)	25	89	2e-09	Gammaproteobacteria	FlgD	NNF51024
	Gene 4 (−, 5993–7279)	–	–	–	–	–	–
	Gene 5 (−, 7442–8089)	38	86	4e-29	*Zymomonas mobilis*	Peptide deformylase	WP013933752
	Gene 6 (−, 8382–9137)	–	–	–	–	–	–
	Gene 7 (−, 9207–9839)	–	–	–	–	–	–
	Gene 8 (−, 9918–10637)	–	–	–	–	–	–
	Gene 9 (−, 11355–12107)	–	–	–	–	–	–
	Gene 10 (−, 12165–12875)	–	–	–	–	–	–
	Gene 11 (−, 13124–13243)	–	–	–	–	–	–
	Gene 12 (−, 13811–14692)	29	65	3e-15	Alphaproteobacteria	ATPase	MBE6446966
	Gene 13 (−, 14689–15390)	47	96	7e-55	Gammaproteobacteria	Orotidine-5′-phosphate decarboxylase	THB68900
	Gene 14 (+, 15539–16942)	38	96	2e-76	*Cetobacterium* sp. 2A	UDP-*N*-acetylmuramate–L-alanine ligase	WP185880408
II (351416 bp – 381560 bp)	Gene 339 (+, 351416–352879)	–	–	–	–	–	–
	Gene 340 (−, 353415–353621)	–	–	–	–	–	–
	Gene 341 (+, 354117–355283)	–	–	–	–	–	–
	Gene 342 (+, 355603–355698)	–	–	–	–	–	–
	Gene 343 (−, 356189–357490)	–	–	–	–	–	–
	Gene 344 (+, 357621–357752)	–	–	–	–	–	–
	Gene 345 (+, 357780–358070)	–	–	–	–	–	–
	Gene 346 (−, 358643–359392)	59	98	8e-21	Rhizobiales	Sulfite exporter TauE/SafE family protein	NRA87956
	Gene 347 (−, 359383–360141)	53	97	2e-95	*Dongia* sp.	Molybdopterin-synthase adenylyltransferase MoeB	WP028099426
	Gene 348 (−, 360144–360482)	30	96	3e-06	*Rhodocyclus purpureus*	Hypothetical	WP201210302
	Gene 349 (−, 360479–361021)	40	95	6e-34	Rhizobiales	Molybdenum cofactor guanylyltransferase	NRA87676
	Gene 350 (−, 361018–361482)	68	100	2e-75	Rhizobiales	Molybdenum cofactor biosynthesis protein MoaE	NRB15406
	Gene 351 (−, 361486–361764)	44	92	2e-17	*Cohaesibacter marisflavi*	MoaD/ThiS family protein	WP090075376
	Gene 352 (−, 361740–362990)	53	94	3e-146	*Stylophora pistillata*	Hypothetical	XP022778573
	Gene 353 (−, 362990–364033)	57	96	2e-136	*Paraferrimonas* sp.	Bifunctional molybdenum cofactor biosynthesis protein MoaC/MoaB	WP163933552
	Gene 354 (−, 364036–365046)	59	96	1e-142	Rhodobacteraceae	GTP 3′,8-cyclase MoaA	MBL4767473
	Gene 355 (+, 365385–367022)	70	100	0	*Exilibacterium tricleocarpae*	Antiporter	WP142928971
	Gene 356 (+, 367044–370799)	82	99	0	Pelagibacteriales	Nitrate reductase	ANY93556
	Gene 357 (+, 370802–372391)	83	98	0	Pelagibacteriales	Nitrate reductase	ANY93557
	Gene 358 (+, 372388–373089)	62	97	9e-98	Pelagibacteriales	Nitrate reductase	ANY93558
	Gene 359 (+, 373086–373787)	75	98	1e-127	Pelagibacteriales	Nitrate reductase I gamma-subunit	ANY93559
	Gene 360 (−, 374111–376684)	31	97	1e-117	*Vibrio* sp.	Hydrogenase expression protein	WP094129926
	Gene 361 (+, 377151–377618)	–	–	–	–	–	–
	Gene 362 (+, 377619–377843)	–	–	–	–	–	–
	Gene 363 (+, 378328–378492)	–	–	–	–	–	–
	Gene 364 (+, 378568–378753)	44	81	3e-06	*Massilia* sp.	NADH pyrophosphatase	KQQ88665
	Gene 365 (+, 378842–378964)	57	100	2e-08	*Treponema ruminis*	NAD(+) diphosphatase	WP184659397
	Gene 366 (+, 379124–379384)	37	90	4e-07	*Legionella quateirensis*	MFS transporter	WP058475153
	Gene 367 (−, 379398–379511)	–	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

Genomic Segment	Protein coding gene [−/+ strand, region(bp)]	BLASTP Similarity			NCBI hit and protein function		
		Similarity (%)	Coverage (%)	e-value	taxa	gene	accession
	Gene 368 (+, 379548–379694)	–	–	–	–	–	–
	Gene 369 (+, 380004–381560)	34	97	1e-105	*Legionella quateirensis*	FAD-dependent monooxygenase	WP058475148
III (509795 bp – 522550 bp)	Gene 495 (+, 509795–510601)	31	44	0.033	*Aurantiacibacter arachoides*	OmpA family protein	WP131453067
	Gene 496 (+, 510893–511840)	–	–	–	–	–	–
	Gene 497 (+, 511908–512690)	–	–	–	–	–	–
	Gene 498 (+, 512867–513706)	–	–	–	–	–	–
	Gene 499 (+, 513999–515030)	–	–	–	–	–	–
	Gene 500 (+, 515307–516158)	–	–	–	–	–	–
	Gene 501 (+, 516447–517250)	–	–	–	–	–	–
	Gene 502 (+, 517459–519294)	–	–	–	–	–	–
	Gene 503 (+, 519681–521591)	–	–	–	–	–	–
	Gene 504 (+, 522218–522550)	35	73	0.001	*Campylobacter pinnipediorum*	Preprotein translocase subunit SecG	WP078423910

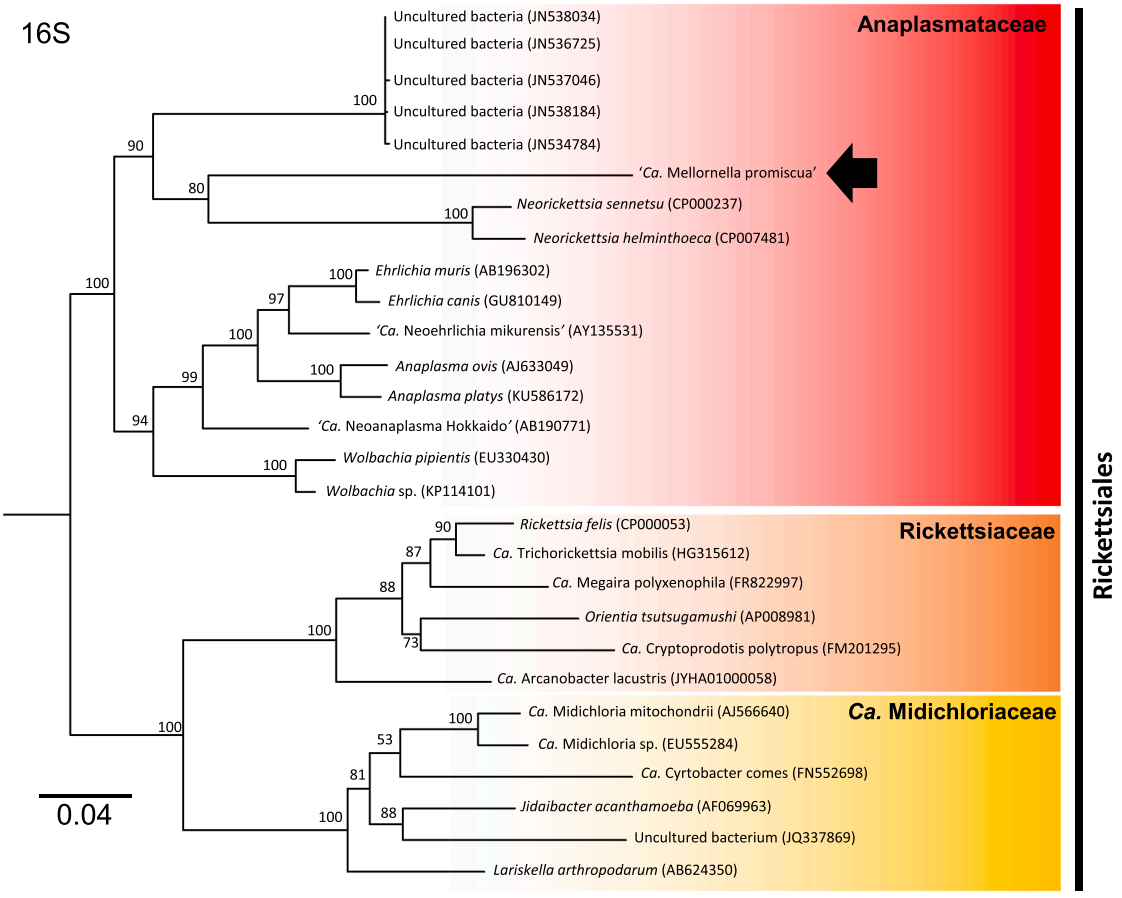

Fig. 4. 16S maximum-likelihood phylogenetic tree of representative species and environmental isolates from across the Rickettsiales. The new species described herein is identified using a black arrow. The represented bacterial families are each also presented in text and colour: Anaplasmataceae (red); Rickettsiaceae (orange); *Ca.* Midichloriaceae (yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

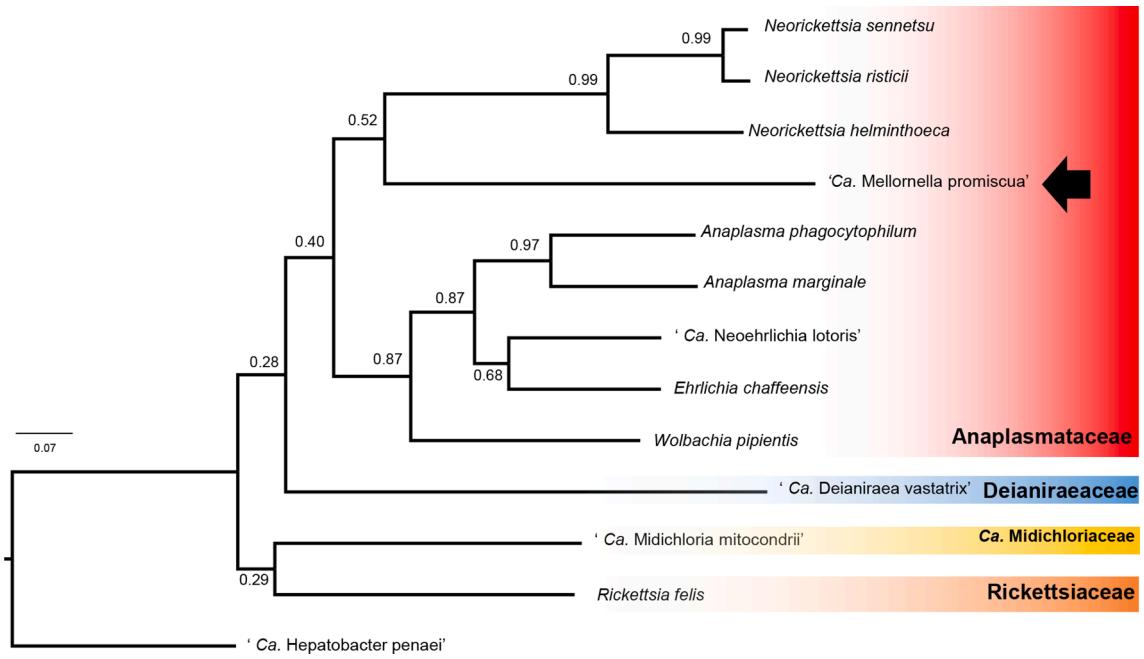

Fig. 5. A STAG-inferred phylogenomic species tree developed from all resulting orthogroup proteins available for all 13 of the species represented, produced in OrthoFinder v2.5.2. The numbers at the nodes reflect bipartition support (0.0–1.0) units to two decimal places, relating to confidence in the position of the branch. Protein sequences were acquired from the following genomic accessions: NC_012026; NC_021880; NZ_CP029077; NZ_JQAJ00000000; NC_015722; NZ_LANX00000000; NZ_CP007480; NZ_CP007481; NC_013009; NC_007798; NZ_JSEL00000000; NZ_CP050531. The bacterial families are each represented by a colour: Anaplasmataceae (red); Deianiraeaceae (blue); *Ca.* Midichloriaceae (yellow); Rickettsiaceae (orange). '*Candidatus* Hepatobacter penaei' is used as an outgroup to root the tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Description of 'Candidatus Mellornella' n. gen. Bojko, McCoy, and Blakeslee 2022

Intracellular bacterial organisms that can act as pathogens of brachyuran hosts. The bacteria form cellular vacuoles in the host hepatopancreas in which they develop, and the plaques are visible using histology. High numbers of sex-pili-expressing bacteria are visible under electron microscopy, some of which display a fibrous inclusion body. Their developmental cycle includes standard binary fission, progressing through an expansion of the bacterial cytoplasm followed by a separation phase. Externally, the animal may not be visibly infected. Members of this genus should be considered based on the availability of genetic/genomic data that shows relative similarity to the type species: '*Ca.* Mellornella promiscua'.

4.3. Description of 'Candidatus Mellornella promiscua' n. sp. Bojko, McCoy and Blakeslee 2022

Members of this species infect the flatback mud crab, *Eurypanopeus depressus,* in marine and brackish habitats on the coastline of the eastern USA. The bacteria are intracellular within the host hepatopancreatocytes and develop within a vacuole of their own creation. Each bacterium measures 0.591 ± 0.075 μm (SD; n = 20) in width and 0.731 ± 0.088 μm (SD; n = 20) in length. To be adequately assigned to this species, genomic data should show high similarity to the genome provided in this manuscript.

Type host: *Eurypanopeus depressus* (common name: flatback mud crab).

Scientific name: The genus '*Ca.* Mellornella' is derived from Rita Mellor, Barry Mellor and Sonia Mellor who are continuously supporting the field of aquatic pathogen discovery. The species name '*Ca.* Mellornella promiscua' is derived from the abundance of apparent sex pili, observable via electron microscopy of the bacterial vacuoles.

Type locality: North Carolina (USA) (34°42′25.06″N; 76°45′6.84″W).

Site of infection: Intracytoplasmic infection of the hepatopancreas.

Type material: Genomic data are reposited in GenBank (Accession Number: CP048228). No cultures are available of this bacterial species; however, original material is stored in the Blakeslee Laboratory (East Carolina University).

5. Discussion

Panopeid mud crabs boast a diversity of parasitic and pathogenic associations (Bojko et al. 2021); however, '*Ca.* Mellornella promiscua' is the first hepatopancreatic rickettsia-like organism (RLO) detected in a panopeid crab. This novel bacterium represents the type species of a novel genus ('*Ca.* Mellornella'), which phylogenetically groups within the Anaplasmataceae (Rickettsiales). This discovery represents the first formally described Rickettsiales from a decapod crustacean (Decapoda) and is one of two rickettsial genera that have been identified to infect crustaceans ('*Ca.* Mellornella', *Wolbachia*) (Cordaux et al. 2001; Werren et al. 2008). Below, we explore the taxonomy and pathology surrounding this new species, including how this novel bacterium may integrate into the surrounding disease ecology of the south-eastern USA coastline. Finally, this discovery may aid in our understanding of bacterial pathogenesis and evolutionary diversity in crustaceans and highlight a new lineage of pathogenic bacteria that may be emerging in crustacean populations.

5.1. Taxonomy and understanding of 'Ca. Mellornella promiscua'

We identify and characterise a novel bacterial pathogen of the coastal panopeid crab, *E. depressus*, using histological, TEM and genomic analytical methods. This novel bacterium is named under the candidate genus and species: '*Ca.* Mellornella promiscua', under the basis that it is dissimilar from any other bacterium described to date but has not yet been determined to be culturable. Using the complete genome, both single gene (16S) and orthogroup-based phylogenetic analyses (Figs. 4 and 5) suggest that this bacterium requires a novel genus and species

within the Anaplasmataceae, forming a long-branch sub-group separate from, but alongside, three *Neorickettsia* isolates. This group contains bacterial symbionts known to cause disease in multiple host species, including humans, and are often vectored by arthropods (Rikihisa, 2006). Despite knowledge of the cytology of the host-pathogen relationship at the genomic and cytological level, it is yet to be determined whether the newly identified '*Ca.* Mellornella promiscua' can cause mortality in the crab host.

Our observational data show that this bacterium causes cytoplasmic hypertrophy and hepatopancreatic degradation in infected host hepatopancreatic cells (Figs. 1 and 2), creating a vacuole in which to develop and divide, in a similar fashion to the somewhat related *Neorickettsia helminthocoeca* (Fischer et al. 2017), but at a greater scale within the cell. TEM sections further highlight the abundance of bacteria in each cell (Fig. 2). To our knowledge, no observable immune response is triggered due to the presence of these bacteria and all histology-prepped specimens were free from any melanisation or granuloma formation in the hepatopancreas.

The genome of the bacterium provided a useful resource to compare against other crustacean pathogens and related Anaplasmataceae, whilst providing insight into possible virulence genes that provide more genetic detail to the pathology we observe. Seventeen potential virulence genes provide us with greater certainty that this bacterium has the capacity to be pathogenic. Examples of these genes include three VirB2 family proteins and a YihY virulence factor; both gene orthologues are common among parasitic Anaplasmataceae (Rikihisa et al. 2010). A third gene encodes a type II toxin-antitoxin system (RatA) protein that may represent the capacity for '*Ca.* Mellornella promiscua' to produce toxins in host cells (Unterholzner et al. 2013).

Most of the genome encoded predicted/hypothetical proteins with greatest amino acid and nucleotide similarity to members of the Rickettsiales (Fig. 3). These include the core genes of this bacterial group. Downstream analysis using antiSMASH suggests the potential production of three secondary metabolites. Naphthocyclinone: a chemical with activity against gram-positive bacteria (Brünker et al. 1999) and possible pigment (Zhu et al. 2020). Moenomycin (phosphoglycolipid): an antibiotic against gram positive bacteria (Halliday et al. 2006) and inhibitor of the transglycosylation process at the final stage of peptidoglycan biosynthesis (cell wall biosynthesis). Finally, Viguiepinol (3-Hydroxypimara-9(11),15-diene) (AKA: diterpene): a chemical related to Furaquinocin (antitumour drug) (Kawasaki, 2006) is part of a diverse chemical group that generally act as flavours, antibiotics, and plant hormones.

Aside from gene orthologues that associate to other Rickettsiales, our analysis of the bacterial genome also revealed a paleovirological history with the *Caudovirales*, as well as encoding three main genomic regions that were highly dissimilar from other Anaplasmataceae (Table 2). Regions I and III were largely dissimilar from other known bacterial genes and may be specific to members of the '*Ca.* Mellornella' genus; however, region II showed high continuity and similarity to a nitrate reductase pathway in Pelagibacteriales as well as Molybdenum/Molybdopterin cofactor synthesis most similar to pathways found in the Rhizobiales (Table 2) – observation of these gene clusters may highlight horizontal transfer of genes between this bacterium and other bacterial groups.

By encoding virulence genes as well as having the capacity to produce antibiotic-like secondary metabolites, we predict that this bacterium may have the capacity to alter the host microbiome to benefit its own growth and reproduction as well as utilize host resources to grow and develop. The pathology and genome of this bacterium support its position as a new member of the Anaplasmataceae, highlighting its intracellular lifecycle and majority gene/amino acid similarity to existing Anaplasmataceae members.

5.2. Opportunities to study 'Ca. Mellornella promiscua'

'*Ca.* Mellornella promiscua' is the first formally identified member of the Anaplasmataceae from a decapod host. Much of the (post-genetic, morphologically derived) diversity relating to the Rickettsiales in decapods remains unconfirmed using genomic data (Bonami and Pappalardo, 1980; Johnson, 1984; Meyers and Shorts, 1990; Messick, 1998). Potentially related bacteria from *Carcinus mediterraneus*, *Callinectes sapidus*, and *Lithodes aequispina* are some that still require such confirmation. More recently, *C. mediterraneus* (also known as: *Carcinus aestuarii*) was found in Argentina displaying an RLO of the hepatopancreas and provides an opportunity to determine whether this bacterium is also a member of the Rickettsiales (Frizzera et al. 2021). While both *C. sapidus* and *L. aequispina* have also been studied recently for endosymbionts, data on their bacterial diversity remains to be explored in detail to characterise any Rickettsiales infections (Shields et al. 2003; Noever et al. 2016; Zhao et al. 2020). Further study into these, and other, crustacean hosts may reveal the same result or uncover further diversity, as we have seen in *E. depressus* collected from the wild.

The small size and cultivability of *E. depressus* provides an easy and sustainable model system to explore the effect of '*Ca.* Mellornella promiscua' on this animal's behaviour and physiology. *Eurypanopeus depressus* and other related panopeid crabs in the ecosystem (e.g., *Rhithropanopeus harrisii* and *Dyspanopeus sayi*) (Jennings et al., 2021), are easily maintained in large numbers in standard laboratory conditions (Tepolt et al. 2020; Blakeslee et al. 2021) and could be further investigated for the potential impacts of the bacterium on crab populations and communities. Given that this new species represents a potential emerging lineage of the Rickettsiales, it seems pertinent to explore whether it can induce lethal or sublethal effects in crustacean hosts, including those utilized in aquaculture/wild-caught fisheries. A further possibility includes exploration of disease transmission, spread, and mitigation in this manageable culture system. For example, a microvirus (*Jodiemicrovirus-1*) has recently been identified from this crustacean's pathobiome and may have the capability to infect '*Ca.* Mellornella promiscua' (Bojko et al. 2019). Using this microvirus in this crustacean-bacterium system could provide us with an easily managed mesocosm experiment to understand virally mediated bacterial control in marine crustaceans.

5.3. Disease ecology of the flat-back mud crab, Eurypanopeus depressus

We identified a significant association between this bacterium and the presence of *L. panopaei*. In total, 7/65 (10.8%) of crab hosts were infected with '*Ca.* Mellornella promiscua', of which 6/7 (85.7%) of crabs were also co-infected with *L. panopaei*, based on histopathological/gross observation. The significant association between rhizocephalans and bacteria, suggests that the presence of the parasite could predispose crabs to become infected with '*Ca.* Mellornella promiscua'. The ecological interactions between these three species (crab, barnacle, bacterium), and possibly the *Jodiemicrovirus*, should be studied in more detail. Much is currently known about the barnacle-crab association, providing us with a strong experimental foundation.

Eurypanopeus depressus is a valuable study organism pertaining to emerging diseases and their spread in North American estuarine and coastal communities. It has significant ecological importance in estuarine food webs that include commercially valuable species like blue crabs (*C. sapidus*) and eastern oysters (*C. virginica*; Williams 1984). Moreover, it is a vector for the transmission of many parasites and diseases (Kroft and Blakeslee 2016; Moore et al. 2020). Most notably, *E. depressus* has been the subject of several studies over the last few decades examining the influence of *L. panopaei* on host physiology, behaviour, ecology, and evolution in Atlantic estuaries (Hines et al. 1997; Tolley et al. 2006; Kruse et al. 2012; O'Shaughnessy et al. 2014; Toscano et al. 2014; Belgrad and Griffen, 2015; Gehman et al. 2017; Tepolt et al. 2020; Brothers and Blakeslee, 2021).

Loxothylacus panopaei is a "body-snatching" parasite that causes permanent castration, eliminating individual fitness and exerting a strong selective pressure (Alvarez et al. 1995). These crab "zombies"

serve as vessels for the barnacle's reproduction (Walker et al. 1992; Alvarez et al. 1995; Carlton et al. 2011). *Loxothylacus panopaei* is native to the Gulf of Mexico and southeast Florida but was absent from most Atlantic populations of mud crab until the middle of the 20th century, when it invaded the Chesapeake Bay in association with Gulf of Mexico oysters (Van Engel et al. 1966). The parasite then spread into mud crab populations in Virginia, the Carolinas, Georgia, northeast Florida, and most recently New York (Hines et al. 1997; Kruse and Hare 2007; Freeman et al. 2013).

The association between '*Ca.* Mellornella promiscua' and *L. panopaei* may relate to a possible ecological/parasitological relationship between these two organisms. Pathogenic bacteria and rhizocephalan parasites have been identified in *Scylla* spp. mud crabs (Portunidae) in India; however, it is unclear whether there is an association between the bacteria and the barnacle (Jithendran et al. 2010). There is a concern that *Scylla* spp. could transmit diseases, like pathogenic bacteria, among aquaculture facilities and to the wild, and so discerning potential transmission vectors is important. In our system, the association we found between barnacle and bacterium co-infection may indicate that *L. panopaei* is a potential carrier of the bacterial pathogen, since it is also a crustacean and could serve as a competent host. Alternatively, infection with *L. panopaei* could increase host susceptibility due to the mechanical damage to the crab, or because the immune system is weakened by rhizocephalan infection. Screening and experimentation is needed to determine why the association we observe exists and what it may mean for estuarine and coastal communities, particularly at sites where *L. panopaei* prevalence is high.

Ecological detail for some Anaplasmataceae has been gathered. All Anaplasmataceae genera vectored by arthropods, such as the *Anaplasma*, *Neoehrlichia* and *Ehrlichia*, result in infections in mammals and their associated vector ecology has been explored in detail (Rymaszewska and Grenda, 2008; Thomas et al. 2009; Derdáková et al. 2014). The *Neorickettsia*, a closer relative to '*Ca.* Mellornella', primarily infect digenean trematodes, which are parasites of vertebrates, and can be vectored by the trematodes they infect (Vaughan et al. 2012). These examples suggest that vector-borne transmission of the Anaplasmataceae may be common in other parasites (i.e. acting as hyperparasites), and this may be the case for the transmission of '*Ca.* Mellornella' in the crab and barnacle system that we observe.

One genus in the Anaplasmataceae appears not to transmit via a vector, but has instead evolved to alter host sex, transmitting vertically in most instances - the *Wolbachia* (Werren et al. 2008). However, horizontal transmission of *Wolbachia* can also occur between arthropod hosts (Stahlhut et al. 2010). This bacterial genus includes members that do not appear to infect vertebrate hosts. Our genomic comparison data suggest that several of the '*Ca.* Mellornella' genes show closest similarity to *Wolbachia* sp.; however, our phylogenetic data support a closer evolution with the *Neorickettsia*. These examples from the Anaplasmataceae ecology literature provide reason to believe that the new bacterium may be vectored and/or has the capacity to transmit both vertically and horizontally. Greater understanding using our model will help to determine how this novel bacterium transmits between hosts, possibly using *L. panopaei* as a vector.

5.4. Conclusions and future aims

This study introduces a new bacterial pathogen, '*Ca.* Mellornella promiscua', within the Anaplasmataceae that infects an ecologically relevant mud crab (*E. depressus*) at 10.8% prevalence and has a statistically significant association with co-infection by the rhizocephalan parasite, *L. panopaei*. Discovery of this new bacterial pathogen highlights a second lineage of the Anaplasmataceae that can cause infection in crustacean hosts, perhaps constituting an emerging group with relevance to crustacean fisheries and aquaculture.

It seems pertinent to explore the disease ecology surrounding the mud crab pathobiome, which is composed of: *L. panopaei*, '*Ca.* Mellornella promiscua', and *Jodiemicrovirus-1* (Bojko et al. 2019). Considering that bacterial pathogens have been associated with other parasites of crustaceans, including some of the taxa described above, it is imperative to further elucidate the relationships of macroparasites as potential vectors of pathogen transmission. Screening methods, such as specific molecular diagnostics, require designing, validation and application to potential host groups (cohabitants: *R. harisii*, *D. sayi*; predators: *C. sapidus*, *M. mercenaria*), to provide a detailed understanding of parasite and pathogen prevalence, and host range. Existing literature on mud crab disease ecology and Anaplasmataceae ecology suggest that the new bacterium may be vectored, providing impetus to explore the statistical relationship we have identified between *L. panopaei* and '*Ca.* Mellornella promiscua' in greater experimental and diagnostic detail.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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