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A novel bacterial infection of the edible crab, *Cancer pagurus*

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Abstract

There are few reports of bacterial diseases in crabs. A juvenile edible crab (*Cancer pagurus*) with a rickettsial-like infection was found in the intertidal zone at Freshwater East in South West Wales in July, 2012. Large numbers of bacteria-like particles were found in the haemolymph and within fixed phagocytes of the hepatopancreas. Molecular sequencing and subsequent phylogenetic analysis showed that the infectious agent was a member of the order Rhizobiales and therefore distinct to bacteria classified as rickettsia.

Key words: Bacterial septicaemia; crustacean; rickettsia-like infections

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1. Introduction

Crustaceans are subject to a range of diseases caused by bacteria (Wang, 2011). While there are many reports of such diseases in some crustaceans such as shrimp (e.g. Austin, 2010) fewer reports have been published on these conditions in brachyuran crabs (Wang, 2011). Although the haemolymph of crabs often contains small numbers of viable bacteria (e.g. Welsh and Sizemore, 1985) their potential to cause sepsis is unknown (Smith et al., 2014).

Rickettsia and rickettsia-like organisms have been widely reported in a variety of crustaceans (e.g. Johnson, 1984; Edgerton and Prior, 1999; Cordaux et al., 2007; Nunan et al., 2013) and in a small number of brachyuran crabs including *Carcinus aestuarii (mediterraneus)* (Bonami and Pappalardo, 1980) and the European shore crab, *C. maenas* (Eddy et al., 2007). Rickettsia are Gram-positive, obligate, intracellular parasites and their inability to grow in standard bacteriological media has been used as evidence of their rickettsial-like nature but without any reliable approach to identification. Therefore, some of these aetiological agents, originally described as “rickettsia-like”, may not belong to the order Rickettsiales (Wang et al., 2004; Wang 2011). An exception to this is the finding that necrotizing hepatopancreatitis, a serious condition of shrimp, is caused by a newly-described bacterium, *Candidatus* Hepatobacter penaei, a member of the order Rickettsiales (Nunan et al., 2013).

This paper reports on a novel rickettsia-like infection found in a juvenile edible crab, *Cancer pagurus,* recorded as part of a wider disease survey already reported (Thrupp et al., 2015).

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2. Materials and Methods

The infected edible crab (an intermoult female of 44 mm carapace width) was collected from Freshwater East, South West Wales, U.K. (51° 39′ 0″ N, 4° 52′ 0″ W; Grid Reference SS016984) in July 2012 as part of a wider survey of disease prevalence (Thrupp et al., 2015). It was transported back to the laboratory in damp seaweed, placed in a sea water aquarium and examined *ca*. 24 hr post-collection.

Haemolymph was collected aseptically from a jointed area at the base of a pereiopod and *ca*. 50 µl was examined using phase contrast microscopy. A further 100 µl of haemolymph was added to 100 µl of sterile marine saline (see Smith et al., 2014 for composition) and aliquots spread onto Petri dishes containing either tryptone soya agar (TSA + 2% sodium chloride) or thiosulphate citrate bile sucrose agar (TCBS +1% sodium chloride) (Becton Dickinson, Oxford, UK). Plates were incubated for 24-48 hr at 25ºC prior to examination. A further 50 µl haemolymph sample was placed in *ca*. 2 ml of ice-cold absolute ethanol and stored at -20 ºC for later 16S rRNA sequence analysis.

The infected crab was injected with Davidson's seawater fixative and left for *ca*. 1 hr. The hepatopancreas, gills and antennal gland were removed and placed in fresh Davidson's seawater fixative. These tissues were left in the fixative for *ca.* 24 hr, after which the tissue was removed and placed in 70% ethanol until later dehydration and embedding in paraffin wax, as detailed previously (Smith et al., 2014). Blocks were sectioned at 7 µm and stained using Cole's haematoxylin and eosin. Sections were examined using an Olympus BX 41 microscope fitted with an Olympus SC30 camera.

To identify the bacteria-like particles observed in the haemolymph, 16S rRNA sequence analysis was undertaken on the infected haemolymph. The sample was

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centrifuged and the pelleted cells extracted and processed with a Qiagen DNeasy® Blood and Tissue Kit; incorporating an initial lysozyme-based disruption step optimized for Gram-positive bacteria, followed by proteinase K digestion as detailed in Whitten et al. (2014). Bacterial 16S rRNA was amplified by touchdown PCR. Cycling conditions were: 3 min at 94°C followed by 33 cycles of 30 sec at 94°C, 30 sec at 61.4°C (-0.7°C/cycle for the first 18 cycles and 49.5°C thereafter), and 30 sec at 72°C, followed by 5 min at 72°C. The primers were 968-f: 5'-ACGCGAAGAACCTTAC-3' and 1401-r: 5'- CGGTGTGTACAAGACCC -3' (Lindh et al., 2008), but with a modified C-rich GC clamp at the 5' end of the forward primer to prevent total strand dissociation during ensuing electrophoresis: 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3'. The resulting amplicons were then profiled using the PCR-temporal temperature gradient gel electrophoresis (TTGE) method to visualize the diversity of bacterial species in the DNA extract. Amplicons from the above PCR reaction were separated by TTGE using the BioRad DCodeTM Universal Mutation Detection System (BioRad Laboratories Inc., Hemel Hempstead, UK) with 7% polyacrylamide gels supplemented with 7.5 mol/L urea and 2% *v/v* glycerol. The optimized running temperature ramped from 55 to 68.6°C at a rate of 0.8°C/h at 69V. The running buffer was 1.25x tris-acetic acid-EDTA buffer. A strong dominant band and a weak minor band was visualized by TTGE, indicating a single dominant bacterial species had infected the haemolymph. This band was excised and the DNA within was amplified by PCR using the above protocol and primers, but lacking the GC clamp. The resulting 403 bp 16S rRNA fragment was sequenced and then screened using the DECIPHER online analysis tool chimeras (http://decipher.cee.wisc.edu/FindChimeras.html; Wright et al. 2012). The terminal

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portion of the sequence was found to contain potential chimeras, so for the purposes of depositing the sequence with NCBI GenBank it was cropped to a chimera-free 276bp fragment. Both the longer (403 bp) and cropped (276 bp) sequences were aligned to 16S rRNA sequences obtained from the NCBI GenBank database, using programmes within the Ribosomal Database Project (RDP Release 11; Cole et al., 2014), and clustered using the RDP2 Tree Builder program. Irrespective of the length of the queried fragment, the resulting closest sequence matches (100% coverage with 98-99% identity) were the same. The 276bp sequence has been submitted to the National Centre for Biotechnology Information (NCBI) GenBank database under accession number KM083124.

3. Results and Discussion

As part of a year-long survey of the disease status at two sites in Pembrokeshire (West Wales, U.K.) a total of 184 crabs were examined from Freshwater East and 182 from Pembroke Ferry. The disease status of these crabs, in terms of those conditions of high prevalence, has already been published elsewhere (Thrupp et al., 2015). Of the crabs surveyed at Freshwater East, one juvenile, intermoult animal collected in July from this site was found to have large numbers ($>10^8$.ml) of bacteria-like particles free in the haemolymph (Fig. 1A). No other crabs collected at the other site at Pembroke Ferry during the same timescale showed any evidence of a similar infection. Furthermore, a large year long survey of the disease status of juvenile edible crabs at two locations in the Gower Peninsula (South Wales, U.K.) failed to observe of this infection in any of the *ca.* 1000 individuals examined (Smith & Rowley, unpublished observations). Thus, we conclude that this disease is relatively rare in populations of juvenile edible crabs in this region of the U.K.

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Attempts to culture the bacteria found in the single infected crab on either TSA or TCBS failed. Histological examination of the hepatopancreas showed the presence of these bacteria-like particles within swollen fixed phagocytes associated with the arterioles in the interstitial space (Figs. 1B - D) and large numbers free in the haemolymph spaces. There was evidence of a host response to the presence of these particles, including nodule formation, in the tissues examined (Fig. 1E). The infected crab also had a co-infection by *Hematodinium* (not shown). TTGE analysis of the bacterial 16S rRNA fragments amplified from the infected crab's haemolymph revealed one dominant band, suggesting a single species of bacteria was responsible for the infection. BLAST / RDP2 analysis of the resulting 403 bp 16S rRNA sequence, and of the cropped 276 bp version of the sequence, identified the bacterium as a member of the alphaproteobacteria, order Rhizobiales, with close homology to members of the Bartonellaceae and Phyllobacteriaceae (Fig. 2), with highest sequence homology to three sequences from uncultured bacteria isolated from spiny lobsters, penaeid shrimps and shore crabs with "milky disease" (GenBank: HQ130337.1; HQ130336.1 and GU947658.1; all 100% coverage with 98-99% identity, irrespective of the length of the queried sequence).

This study provides the first report of a bacterial infection of edible crabs, similar to that observed in a variety of other crustacean hosts albeit at very low levels in the population. Because it was impractical to examine the haemolymph of the crabs at the time of collection while on-shore, we cannot rule out the possibility that the severity of infection seen may have been altered by the stress of transport and collection and the short delay in examination of the haemolymph when back in the laboratory.

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There have been numerous reports of “rickettsia-like” organisms found in various cells and tissues of crustaceans (e.g. Johnson, 1984; Edgerton and Prior, 1999; Cordaux et al., 2007; Nunan et al., 2013). Some of these infect the cells of the hepatopancreatic tubules (Edgerton and Prior, 1999) while others, like those in the current report, parasitize the fixed phagocytes within the interstitial space between these tubules (e.g. Eddy et al., 2007). The causative agent of the condition seen in the edible crabs in the present study does not appear to be a member of this group, as the phylogenetic analysis places it within the order Rhizobiales. It also shares homology and similar pathology to the unidentified bacterium that is responsible for “milky disease” in the shore crab, *C. maenas* (Eddy et al., 2007). Whether this bacterium can infect both edible and shore crabs is unknown but these animals share a similar distribution on-shore. The current study has revealed that care should be taken in assigning the term “rickettsia-like” to infections of invertebrates based simply on the intracellular location of the pathogen and its failure to grow in defined media.

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FIGURE LEGENDS

Fig. 1A-E. (A). Phase contrast micrograph showing bacteria-like particles free in the haemolymph of the edible crab collected in July from Freshwater East. Scale bar = 10 µm. (B). Histological appearance of enlarged fixed phagocytes (Ph) in the interstitial space of the hepatopancreas from the affected crab shown in Fig. 1A. (C). High power micrograph shows the presence of large numbers of bacteria-like particles within these fixed phagocytes. (C). Normal appearance of fixed phagocytes (Ph) in the interstitial space surrounding the tubules (Tu) of the hepatopancreas from an uninfected juvenile edible crab. (E). Low power micrograph of the hepatopancreas of the infected crab showing small numbers of nodules (arrows) in the interstitial spaces between the tubules (Tu). Note lack of damage to tubules. Scale bars = 25 µm (A, B, D), 5 µm (C), 100 µm (E).

Fig. 2. Phylogenetic tree constructed using the partial 16S rRNA sequence amplified from a *C. pagurus* haemolymph sample heavily infected with unculturable bacteria (KM083124). The 16S rRNA sequences were clustered using the RDP2 Tree programme with rRNA sequence from *Spiroplasma eriocheiris* DQ917753 as the out-group. The rRNA sequences from other bacteria were added from RDP2 to give context and these include the closest database matches to the crab unculturable bacteria. (T) = type strain. Scale bar: 10/100 (10%) sequence difference. Inset: TTGE gel bacterial 16S rRNA fragments amplified from the infected crab haemolymph. Different concentrations of the sample were loaded into lanes a-c, and in all cases one dominant band was visualized. M = gel migration markers. The dominant band in lane c was sequenced and used to construct the phylogenetic tree.

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Figure 1

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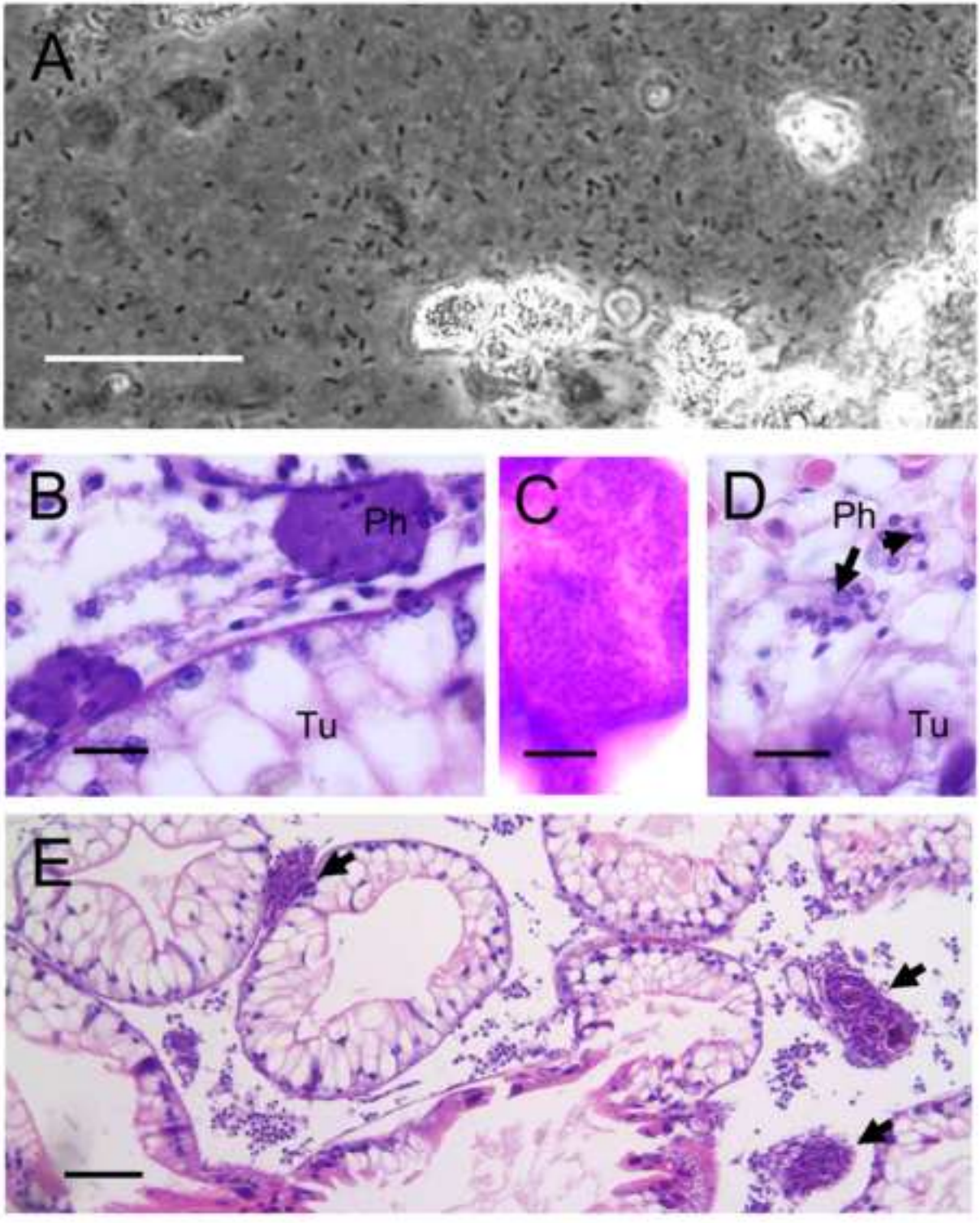

Figure 2

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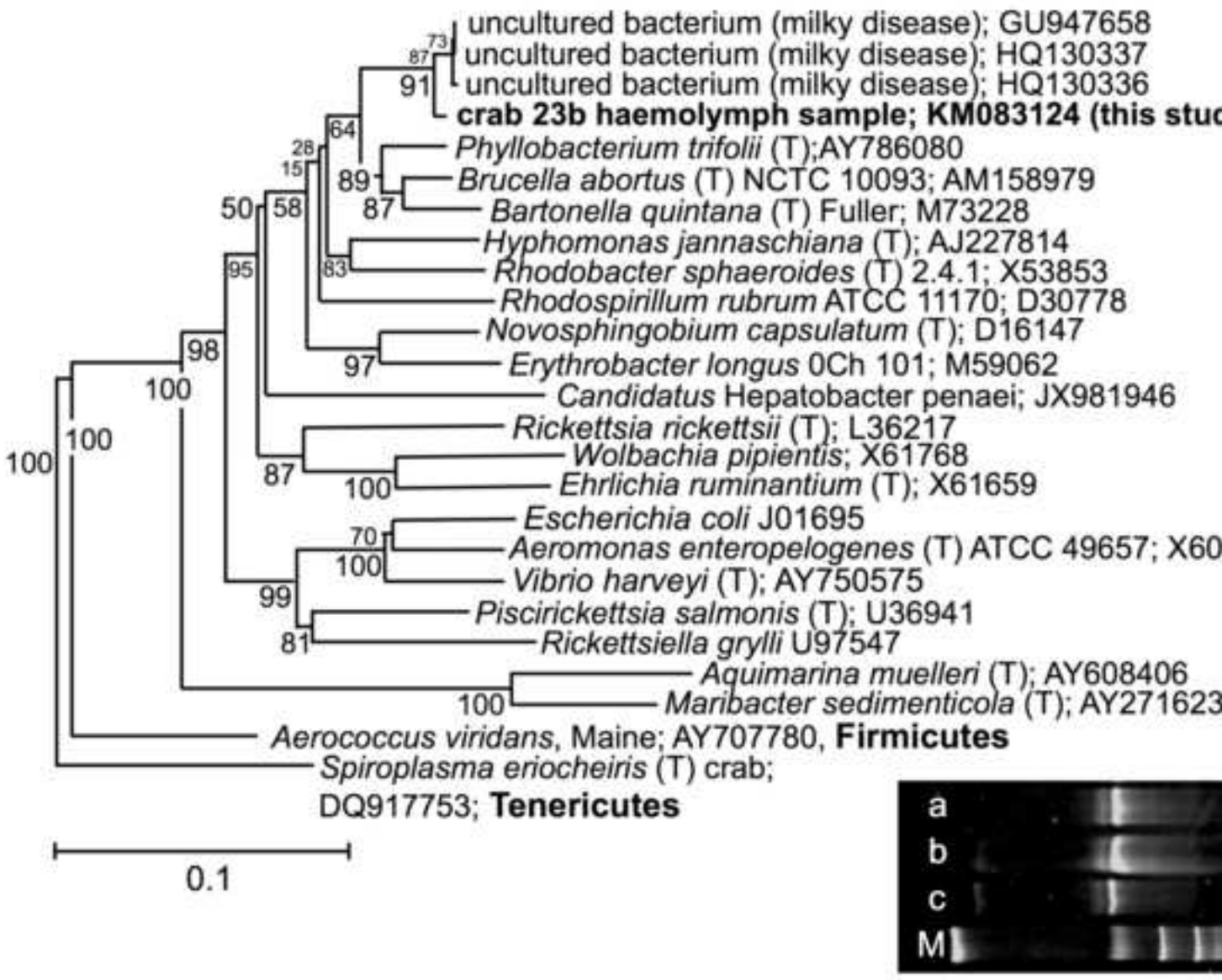
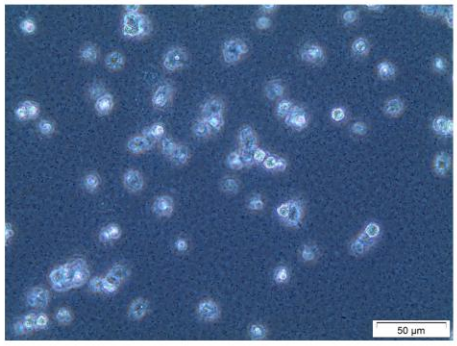


Graphical abstract



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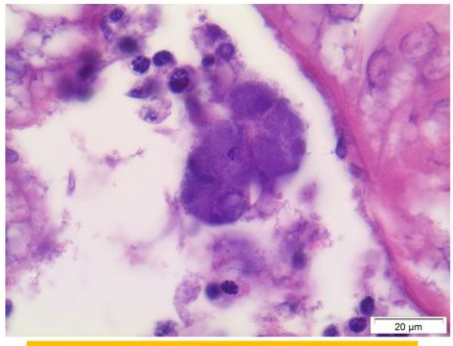
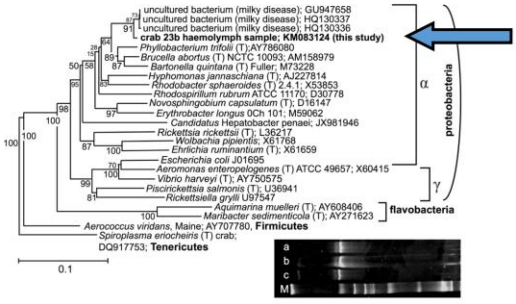


INFECTION OF FIXED PHAGOCYTES IN THE HEPATOPANCREAS



Highlights

This reports a new bacterial disease of the edible crab, *Cancer pagurus*

Infected crabs have large numbers of bacteria-like particles in the haemolymph and fixed phagocytes in the hepatopancreas

The bacterium was found to be similar to the causative agent of “milky disease” in other species of crabs.

Although the pathology is similar to that of rickettsia-like diseases of other crustaceans the causative agent is not a member of the Rickettsiales