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Candidatus Renichlamydia lutjani, a Gram-negative bacterium in internal organs of blue-striped snapper *Lutjanus kasmira* from Hawaii

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ABSTRACT: The blue-striped snapper *Lutjanus kasmira* (Perciformes, Lutjanidae) are cosmopolitan in the Indo-Pacific but were introduced into Oahu, Hawaii, USA, in the 1950s and have since colonized most of the archipelago. Studies of microparasites in blue-striped snappers from Hawaii revealed chlamydia-like organisms (CLO) infecting the spleen and kidney, characterized by intracellular basophilic granular inclusions containing Gram-negative and Gimenez-positive bacteria similar in appearance to epitheliocysts when seen under light microscopy. We provide molecular evidence that CLO are a new member of *Chlamydiae*, i.e. *Candidatus* Renichlamydia lutjani, that represents the first reported case of chlamydial infection in organs other than the gill in fishes.

KEY WORDS: Epitheliocystis · Chlamydia-like organism · *Chlamydiae* · Blue-striped snapper · *Lutjanus kasmira*

INTRODUCTION

The blue-striped snapper *Lutjanus kasmira* (Perciformes, Lutjanidae), or taape, is a widespread tropical marine fish. It was introduced in the 1950s into Oahu, Hawaii, USA, from Tahiti and the Marquesas as a food and sport fish and has since spread throughout the Hawaiian archipelago (Randall 1987). Studies of the microparasites of this fish revealed a high prevalence of splenic coccidia (47%) and small intracellular Gram-negative bacteria (22%) in the kidney, which, based on light microscopy, were compatible in morphology with epitheliocysts (Work et al. 2003). While the coccidia did not seem to cause significant pathological changes, intracellular bacteria stimulated a mononuclear inflammatory response and increased in prevalence with the size of the fish but were absent in the largest size classes. Given their similarity to epitheliocysts based on light microscopy, these organisms were provisionally named epitheliocystis-like organisms (ELO) (Work et al. 2003). However, epitheliocystis in fish typically affects the gill (Nowak & LaPatra 2006), whereas in Hawaii the organisms were in the kidney, and their morphology seen under electron microscopy was not compatible with epitheliocysts, as described by others (Wolke et al. 1970, Groff et al. 1996).

Recently, molecular approaches have confirmed that *Chlamydiae* are the agents in epitheliocysts and various lineages are involved (Draghi et al. 2004, 2007, Meijer et al. 2006, Karlsen et al. 2008). In this study, we used polymerase chain reactions (PCR) and sequencing of 16S rDNA to show that these Gram-negative bacteria-like organisms (BLO) belong to *Chlamydiae*. This represents the first reported case of chlamydial infection in fish affecting the kidneys.

MATERIALS AND METHODS

Fish were collected from Oahu, Hawaii, as part of general surveillance for microparasites in reef fish (Work et al. 2003, 2010). For light microscopy, caudal kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin, Gram (Prophet et al. 1992) or Gimenez (Gimenez 1964). The latter stain is routinely used to confirm chlamydia using light microscopy (Nabeya et al. 1991). Paired samples were preserved in 95% ethanol or DMSO/EDTA/NaCl (Dawson et al. 1998) for DNA extraction. Samples were confirmed as BLO-positive or BLO-negative based on examination of histology slides. DNA was extracted from kidneys of affected (n = 3) and unaffected (n = 3) fishes using a Wizard SV Genomic DNA (Promega) kit following the manufacturer's instructions.

PCR assays were conducted with pan-chlamydia 16S rDNA primers CF1 (5'-CGT GGA TGA GGC ATG CRA GTC G-3'), CR6 (5'- GTC ATC RGC CYY-ACC TTV SRC RYY TCT-3') and CR7 (5'-TAC CTT GTT ACG ACT TMA YCC YAG-3') as described by Corsaro et al. (2002), under reaction conditions of an initial 5 min at 95°C, followed by 40 cycles of 1 min at 94°C, 30 s at 60 or 62°C and 1 min 30 s at 72°C, and a final extension of 10 min at 72°C. Purified PCR products were sequenced with a set of inner primers, and the obtained sequences were compared with GenBank database sequences using the basic local alignment search tool (BLAST) (Altschul et al. 1990). A master alignment, including closest relatives and representatives of the major chlamydial lineages, was produced using ClustalW (Larkin et al. 2007), and phylogenetic analyses were performed by applying analyses for distance (neighbor joining, NJ) and maximum parsimony (MP) with MEGA5 (Tamura et al. 2011) and maximum likelihood (ML, GTR+I+Γ model) with TREEFINDER (Jobb et al. 2004), using 1000 bootstrap replicates. Similarity sequence values were calculated using BioEdit (Hall 1999).

The nearly full-length 16S rDNA sequence of the BLO infecting the blue-striped snapper *Lutjanus kasmira* is available in GenBank under the accession no. JN167597.

RESULTS

Fish affected with BLO showed no evident gross lesions or adverse clinical signs. Based on microscopy, the organisms consisted of variably sized circular clusters of Gram-negative bacteria. In putative early infections, bacteria were surrounded by lymphocytes, whereas in putative chronic infections, bacteria were surrounded by a capsule consisting of collagen mixed with plump fibroblasts. The bacteria stained positive with Gimenez stain and were Gram-negative (Fig. 1).

We initially obtained a 300 base pair (bp) fragment from an ethanol-preserved sample, which showed 85% sequence identity with the uncultured 'rhabdochlamydia' CN808 (GenBank accession no. EU090709), recovered from a human respiratory sample (Haider et al. 2008). PCR from unaffected fish samples were negative or gave non-specific sequences. Nearly full 16S rDNA sequences were subsequently obtained from DMSO/EDTA/NaCl-preserved kidneys originating from 3 infected fish. In a pair-wise sequence analysis without indels, BLO were 91.7% identical with the same phylotype CN808 and 88.2 to 88.7% identical with *Rhabdochlamydia* spp. Similarity values of the BLO and CN808 with other *Rhabdochlamydiaceae* ranged from 88.0 to 90.3%, whereas within the remaining *Rhabdochlamydiaceae*, values ranged from 88.0 to 94.4%. The similarities of BLO were 85 to 86.7% with the sister-group of *Simkaniaceae* and <86% with the other chlamydial lineages. Phylogenetic analysis (Fig. 2) showed that BLO formed a highly supported holophyletic lineage with the clone CN808, basal within the *Rhabdochlamydiaceae*, or intermediate between *Rhabdochlamydiaceae* and *Simkaniaceae*, and distant from the 2 recognized *Rhabdochlamydia* species infecting arthropods (Kostanjsek et al. 2004, Corsaro et al. 2007).

DISCUSSION

The BLO identified in blue-striped snapper had light microscopic and staining characteristics typical of *Chlamydiae* characterized by intracellular inclusions of granular basophilic clusters of bacteria that stained Gram-negative and positive for Gimenez (Gimenez 1964), a histological stain commonly used to identify *Chlamydiae* in vertebrates (Nabeya et al. 1991) and invertebrates (Meyers 1979). This is the first reported case of chlamydia-like bacteria infecting internal organs (kidney and spleen) of fish. A molecular phylogenetic analysis suggests that the novel organism likely represents a new high-ranking taxon within the *Rhabdochlamydiaceae* or intermediate between *Rhabdochlamydiaceae* and *Simkaniaceae*. However, it shows a strikingly different mor-

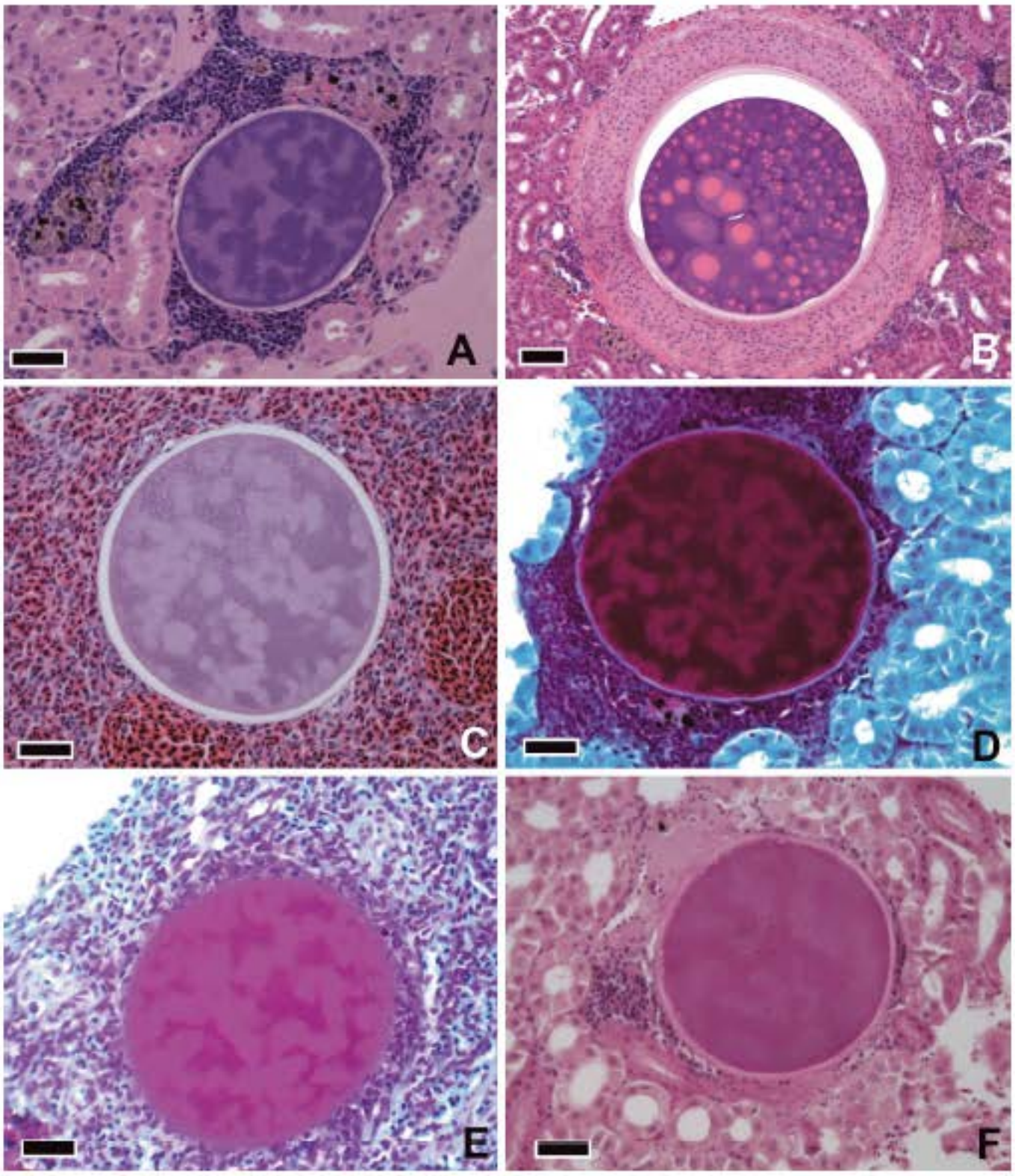

Fig. 1. Photomicrographs of chlamydia-like organism (CLO) in (A,B,D,F) caudal kidney and (C,E) spleen of bluestriped snappers *Lutjanus kasmira*. (A–C) Hematoxylin and eosin, (D,E) Gimenez, (F) Gram. (A,C–F) Putative early infection; note mild lymphoid infiltrates surrounding CLO in (A), (D), and (F). (B) Putative chronic infection; note connective tissue capsule mixed with plump fibroblasts surrounding CLO. In general, for a given stain, organisms in kidneys (A,D) stain darker than in spleen (C,E) for unknown reasons. Scale bar = 50 µm for all photos

phology from the 2 *Rhabdochlamydia* spp. recovered from arthropods, which have elongate rickettsiella-like 5-layered elementary bodies (Kostanjsek et al. 2004, Corsaro et al. 2007). Similarly, based on electron microscopy (Work et al. 2003), BLO differ from epitheliocystis agents infecting gills of fish (Wolke et al. 1970). BLO and *Rhabdochlamydia* are relatively distant genetically, and the closest relative of BLO is the uncultured strain CN808 of unknown morphology recovered from a human respiratory sample (Haider et al. 2008). Moreover, *Rhabdochlamydiaceae* as well as *Simkaniaceae* show a large diversity in putative genus-level phylotypes (i.e. 16S rDNA similarities <95%) with clone PRPR10 from the demosponge *Suberites zeteki* from Hawaii (Zhu et al. 2008) or clones and strains recovered in Europe by protistan coculturing from wastewater and freshwater samples (Horn & Wagner 2001, Corsaro et al. 2002, 2003, 2009, Corsaro & Venditti 2009). Such a large genetic diversity could likely reflect the persis-

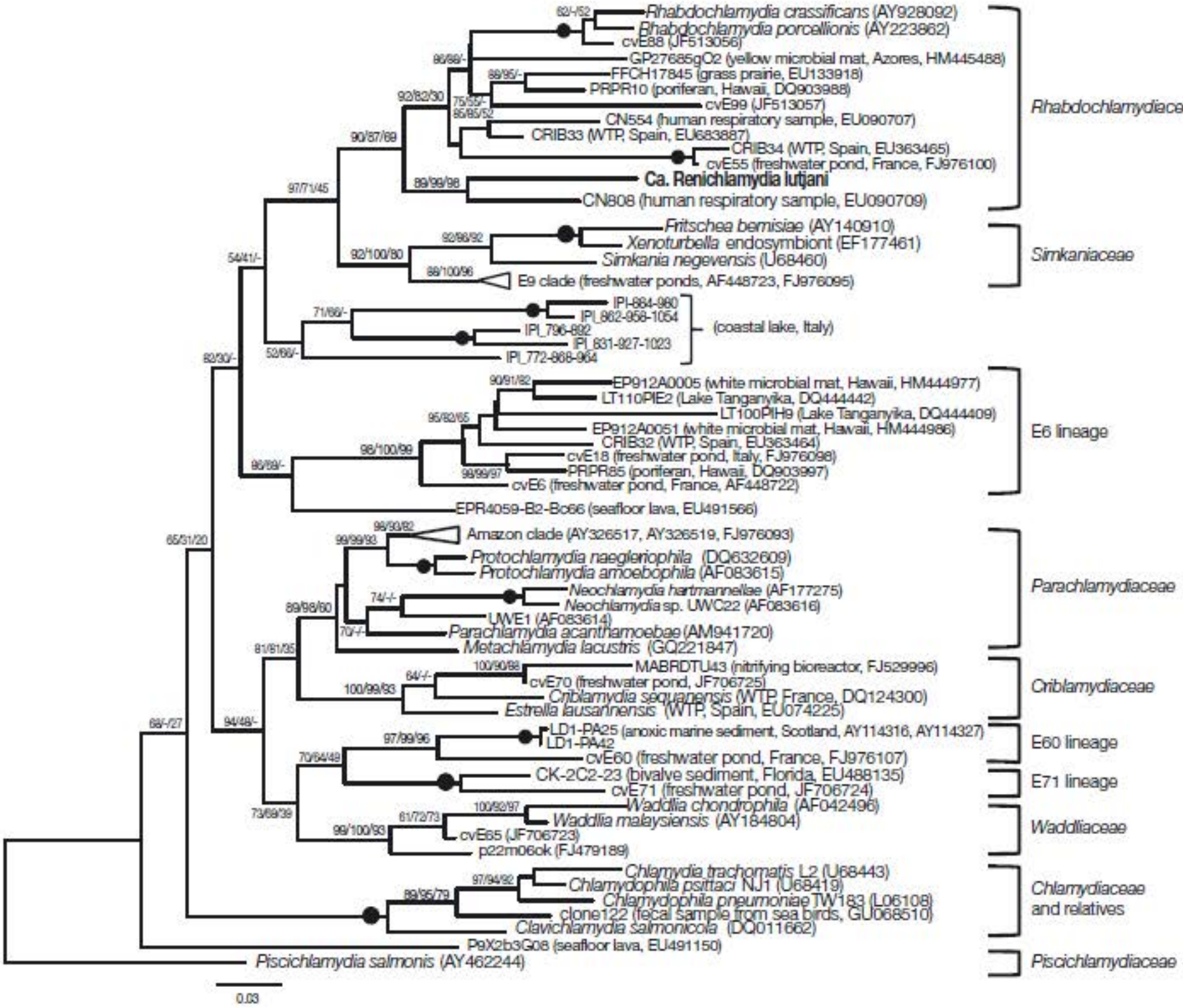

Fig. 2. Maximum-likelihood phylogenetic tree of the *Chlamydiae*. The novel chlamydia *Candidatus* Renichlamydia lutjani retrieved in this study is in bold. Origins and GenBank accession nos. are in parentheses. Numbers at the nodes represent bootstrap values obtained by maximum likelihood (ML)/neighbor joining (NJ)/ maximum parsimony (MP) after 1000 replications; filled circles represent values of 100 % obtained with all 3 methods. WTP: water treatment plant

tence of chlamydia-like morphologies in *Simkaniaceae* and paraphyletically in some *Rhabdochlamydiaceae*, with the development of rickettsiella-like elementary bodies only in the sublineage infecting arthropods.

Since the first description of epitheliocystis by Hoffman et al. (1969), chlamydiae were suggested to be the causative agents based on morphology. This was recently confirmed by molecular approaches targeting the 16S rDNA. Nearly full sequences revealed 2 novel *Chlamydiae* affecting salmonids, *Candidatus* Piscichlamydia salmonis and *Candidatus* Clavochlamydia (= Clavichlamydia) salmonicola (Draghi et al. 2004, 2010, Karlsen et al. 2008), whereas partial sequences revealed the involvement of *Neochlamydia* sp. (*Parachlamydiaceae*) and other phylotypes (Meijer et al. 2006, Draghi et al. 2007, 2010, Polkinghorne et al. 2010) falling within *Rhabdochlamydiaceae* and the E6 lineage (Corsaro & Venditti 2009). *Simkaniaceae* also might be epitheliocystis agents,

with the undescribed 'salmon symbiont' from gills (GenBank acc. no. EU326493).

Epitheliocystis is an infectious disease affecting the gills of several marine and freshwater teleost fishes (Corsaro & Venditti 2004, Nowak & LaPatra 2006), although infections have been occasionally reported in chondrichthyan fishes (Borucinska & Frasca 2002, Polkinghorne et al. 2010). Infection with epitheliocystis is limited to the gills and less commonly the skin and oral cavity. Infections have never been documented in internal organs. In contrast, other pleomorphic intracellular pathogens, i.e. *Piscirickettsia*, *Francisella* (*Gammaproteobacteria*) (Mauel & Miller 2002, Mauel et al. 2003, Mauel et al. 2005) and at least a case of *Neorickettsia* (*Alphaproteobacteria*) (Pusterla et al. 2000), cause systemic infections in fish affecting internal organs like the kidney, liver and spleen. No cyst-like enlarged cells are formed. Our findings thus identify another organism belonging to *Chlamydiae* that infects the internal organs of fish. Demographic studies suggest that this parasite is directly transmitted and may be of limited pathogenicity (Work et al. 2010). Future studies should include solidifying these findings by other methods, such as *in situ* hybridization or immunohistochemistry.

Systematic description

Name: *Candidatus* Renichlamydia lutjani.

Etymology: *Candidatus*, category for obligate intracellular bacteria. *Renichlamydia* n. gen. Reni, L.n. renes, the kidneys; *Chlamydia*, N.L. fem. n., a bacterial genus name; *Renichlamydia* N.L. fem. n., kidney chlamydia. Ca. Renichlamydia lutjiani n. sp., *lutjiani* N.L. gen. sing. n., of *Lutjanus*, genus name of the fish host.

Physical description: Spherical cells 1 to 2 µm in diameter, with crystalloid or electron-dense granules, inside a thin inclusion membrane, surrounded by a granular matrix with mitochondria and an external thick capsule of collagen and fibroblasts (Work et al. 2003).

Pathology: Fish are infected mainly in the kidney, occasionally in the spleen.

Natural host: Blue-striped snapper *Lutjanus kasmira*.

Distribution: Pacific Islands (Work et al. 2010).

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