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Monomorphic pathogens: The case of *Candidatus* Xenohaliotis californiensis from abalone in California, USA and Baja California, Mexico

Francesco Cicala[a], James D. Moore[b], Jorge Cáceres-Martínez[c], Miguel A. Del Río-Portilla[c], Mónica Hernández-Rodríguez[c], Rebeca Vásquez-Yeomans[c], Axayácatl Rocha-Olivares[a,*]

[a] Molecular Ecology Laboratory, Department of Biological Oceanography, CICESE, Carretera Tijuana-Ensenada km 3918, Ensenada, Baja California 22860, Mexico
[b] Bodega Marine Laboratory, University of California at Davis, P.O. Box 247, Bodega Bay, CA, USA
[c] Department of Aquaculture, CICESE, Carretera Tijuana-Ensenada km 3918, Ensenada Baja California 22860, Mexico

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ABSTRACT

Withering syndrome (WS) is a chronic wasting disease affecting abalone species attributed to the pathogen *Candidatus* Xenohaliotis californiensis (*C*Xc). Wild populations of blue (*Haliotis fulgens*) and yellow (*H. corrugata*) abalone have experienced unusual mortality rates since 2009 off the peninsula of Baja California and WS has been hypothesized as a possible cause. Currently, little information is available about the genetic diversity of *C*Xc and particularly the possible existence of strains differing in pathogenicity. In a recent phylogenetic analysis, we characterized five coding genes from this rickettsial pathogen. Here, we analyze those genes and two additional intergenic non-coding regions following multi-locus sequence typing (MLST) and multi-spacer typing (MST) approaches to assess the genetic variability of *C*Xc and its relationship with blue, yellow and red (*H. rufescens*) abalone. Moreover, we used *16S rRNA* pyrosequencing reads from gut microbiomes of blue and yellow abalone to complete the genetic characterization of this prokaryote. The presence of *C*Xc was investigated in more than 150 abalone of the three species; furthermore, a total of 385 DNA sequences and 7117 *16S rRNA* reads from *Candidatus* Xenohaliotis californiensis were used to evaluate its population genetic structure. Our findings suggest the absence of polymorphism in the DNA sequences of analyzed loci and the presence of a single lineage of *C*Xc infecting abalone from California (USA) and Baja California (Mexico). We posit that the absence of genetic variably in this marine rickettsia may be the result of evolutionary and ecological processes.

1. Introduction

The Mexican peninsula contains seven exploitable abalone species; however, the actual harvest is focused almost entirely on blue (*H. fulgens* or Hf) and yellow (*H. corrugata* or Hc) abalone (Morales-Bojórquez et al., 2008; SAGARPA, 2009). Since 2009, unusual mortalities have been observed in wild populations of both species in different fishery areas of southern Baja California (Cáceres-Martínez et al., 2011). Among the possible causes responsible for these unusual mortality events is the existence of *C*Xc strains differing in pathogenicity (Cáceres-Martínez et al., 2011).

Thus far, *C*Xc remains an uncultured bacterium and, until recently, the *16S rRNA* gene was the only known DNA sequence. The few sequences available from this ribosomal gene were insufficient to assess levels of intra-specific variability (Balseiro et al., 2006; Friedman, 2012; Kiryu et al., 2013). Thus, the limited genetic knowledge about *C*Xc genetic variability has been the primary obstacle in understanding its influence on WS pathogenesis. *Candidatus* Xenohaliotis californiensis (*C*Xc) is an obligate Gram-negative intracellular bacterium in the family *Anaplasmataceae* from the order *Rickettsiales* (Cicala et al., 2017; Friedman, 2012; Garrity et al., 2004). It is an intracellular organism, inhabiting the cytoplasm of abalone (*Haliotis* spp.) digestive epithelial cells (Friedman et al., 2000). *C*Xc has been identified as the etiological agent of a chronic disease known as Withering syndrome (WS) (Gardner et al., 1995) or abalone Rickettsiosis (Cáceres Martínez, 2002; Friedman, 2012). Advanced stages of WS include severe morphological and physiological anomalies resulting in physiological starvation, anorexia, catabolism of the food muscle and finally death (Crosson et al., 2014; Friedman et al., 2003; Moore et al., 2001).

The ability to detect genetic structure in pathogenic bacterial populations may provide useful information to understand epizootiological events and trace disease outbreaks (Fournier and Raoult, 2007; Fournier et al., 2003; Maiden et al., 1998). Accordingly, a variety of phenotypic and genotypic methods have been developed for bacterial

* Corresponding author.
E-mail address: arocha@cicese.mx (A. Rocha-Olivares).

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inter and intra-specific characterization (Fournier and Raoult, 2007). Among them, PCR-based DNA sequence-typing analyses are some of the most rapid, reliable and easiest methods to identify bacteria intraspecifically (Fournier and Raoult, 2007; Maiden et al., 1998). Maiden et al. (1998) proposed Multi-locus Sequence Typing (or MLST) as a universal and portable method for characterizing bacteria strains. This method involves the sequencing of several housekeeping gene fragments and defines a bacterial strain in terms of its "sequence type" (or haplotype) consisting in the combined DNA sequences from the different loci (Maiden et al., 1998). Moreover, Fournier et al. (2004) proposed a second approach named Multi Spacer Typing (or MST), which is based on the analysis of intergenic non-coding spacer sequences instead of structural genes. The assumption of MST is that non-coding regions are less subject to selective pressure than housekeeping genes and consequently may accumulate more genetic changes over time within a specific lineage (Fournier and Raoult, 2007; Fournier et al., 2004). Both approaches have been successfully used for inter and intra-specific genetic characterization of different bacterial species (Martino et al., 2011; Santos et al., 2012; Thompson et al., 2005) including some in the order *Rickettsiales* (Baldo et al., 2006; Fournier and Raoult, 2007; Fournier et al., 2004; Zhu et al., 2005).

Recently, we characterized partial sequences from the *16S rRNA*, *23S rRNA*, *ftsZ*, *virB11* and *virD4* genes to resolve the phylogenetic position of *C*Xc among *Rickettsiales* (Cicala et al., 2017). Here, we use these genes under the framework of MLST, adding two intergenic regions, located between *23S* and *5S rRNA* (Genbank access number: KY882033) and *virB11-virD4* (Genbank access number: KY882034) genes, under a MST framework in order to characterize the genetic diversity of *C*Xc at different levels: (*i*) geographically, across exploited areas in southern Baja California, (*ii*) taxonomically, between *H. corrugata* and *H. fulgens* hosts; and (*iii*) epizootiologically, between healthy and morphologically WS affected abalone. Additionally, we also analyzed eight specimens of red abalone *H. rufescens* (or Hr) from northern California, USA, to extend the geographic and taxonomic scope of the survey.

2. Materials and methods

2.1. Sampling, experimental design and DNA extraction

Sampling was carried out in April and November 2012. Wild abalone (Hf and Hc) were collected by divers during commercial fishing operations from eight localities along the Pacific coast of southern Baja California, Mexico (Fig. S1). We followed Friedman (2012) to record the presence/absence of clinical external signs of WS in sampled specimens; for example, color alteration and pedal muscle and mantle retraction. Gastro intestinal (GI) tissue (ca. 30 mg of post-esophagus) was carefully dissected from landed specimens and immediately transferred to sterile 1.5 ml microcentrifuge tubes containing molecular grade ethanol. Additional GI tissue samples were obtained from eight *C*Xc-infected Hr abalone maintained in the UC Davis Bodega Marine Laboratory (California, USA).

Total DNA was extracted from abalone gut tissues using a DNeasy blood & tissue kit (Qiagen, Valencia, CA, USA), following manufacturer's protocols. PCR fragments were produced using published (*23S rRNA*- ITS.1 and *ftsZ* loci (Cicala et al., 2017); and newly designed primers (*16S rRNA*, *virB11*- ITS.2-*virD4*, Table 1).

2.2. Gene selection and PCR amplification

The experimental design involved an initial amplification and sequencing of DNA from at least 3 abalone for each locus (n = 7), species (n = 3) and geographic locality (n = 8 for Hf and Hc, and n = 1 for Hr). In order to increase sample size for the most variable loci additional organisms were analyzed.

The MLST and MST approaches were performed using DNA sequences from five genes (products shown in parentheses): *16S rRNA* (small ribosomal sub-unit), *23S rRNA* (large ribosomal sub-unit), *ftsZ* (a cell division protein), *virD4* (a type IV secretion system protein), v*irB11* (a type IV secretion system protein) and two intergenic non-coding spacer sequences located between the *23S-5S* rRNA (ITS.1) and *virB11-virD4* (ITS.2) genes (Table S1).

Amplifications were carried out in 20 µl reactions containing: 120 ng of DNA, 1X PCR buffer, 1.5 mM $MgCl_2$ (both Kapa Biosystems, Woburn, MA, USA), 0.2 mM dNTPs (New England Biolabs, Beverly, MA, USA), 0.3 µM of each primer, and 1U of *Taq* polymerase (Kapa Biosystems, Woburn, MA, USA). Thermal cycling conditions were: 94 °C for 4 min, followed by 40 cycles of 94 °C for 1 min, with specific annealing temperatures for each locus (Table 1) of 30 s, 72 °C for 30 s, and a final extension for 8 min at 72 °C. For the *virB11*- ITS.2 -*virD4* region, amplicons obtained using the primers VirB11ss3F and VirD4ss2R (Table 1), were subsequently used as DNA template in nested PCRs (combining the primers VirB11ss3F and VirD4ss1R; Table 1), in order to increase PCRs yield or to reduce nonspecific amplification. Nested PCRs, were performed using the same chemistry and thermal cycling conditions mentioned above. Amplicon size and quality were verified by 1.5% agarose gel electrophoresis before enzymatic purification (ExoSAP-IT, USB-Affymetrix, Cleveland, OH, USA) and cycle sequencing using an ABI 3730XL automatic DNA sequencer (Macrogen Inc., Rockville, MD, USA).

2.3. Microbiome sequencing and bio-informatics pipeline

Additional *C*Xc genetic diversity data was obtained from GI tract microbiomes. A fragment of approximately 500 bp of the *16S rRNA* gene was amplified using the universal eubacterial primers 28F (Ludwig et al., 1993) and 519R (Ruff-Roberts et al., 1994) (Table 1) from *H. fulgens* and *H. corrugata* GI tissue samples. The PCR master mix was prepared in a 20 µl reaction of 100 ng of DNA, 1X PCR Buffer (Kapa Biosystems, Woburn, MA, USA), 1.5 mM of $MgCl_2$ (Kapa Biosystems, Woburn, MA, USA), 0.2 mM of dNTPs (New England Biolabs), 0.5 µM of each primer, 0.4 mM of bovine serum albumin (New England Biolabs, Beverly, MA, USA) and 1U of Taq polymerase (Kapa Biosystems, Woburn, MA, USA). Thermal cycling conditions were as follows: an initial denaturation at 94 °C for 4 min, 40 cycles of 94 °C for 1 min; 62 °C for 30 s. and 72 °C for 30 s., and a final extension step of 8 min at 72 °C. Confirmation of amplification was carried out by 1.5% agarose gel electrophoresis.

Amplicons were subject to massive parallel next generation sequencing using bacterial tag-encoded FLX Titanium amplicon pyrosequencing (bTEFAP) following Dowd et al. (2008) (Research and Testing Laboratory, Lubbock, TX). The *16S rRNA* raw reads were analyzed using the software package Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010a, 2010b). Initial analyses included demultiplexing. Next, reads were filtered using quality scores generated during pyrosequencing according to the following QC criteria: (*i*) minimum and maximum length settled at 250 and 550 bp, respectively; (*ii*) the default minimum quality Phred score was set at 25; (*iii*) no mismatches in primers and barcode sequences were allowed; (*iv*) maximum homopolymer length was set at 6 bp. After QC tests, the remaining sequences were clustered into operational taxonomic units (OTUs) at 99% sequence similarity using the UCLUST algorithm (Edgar, 2010). We chose the longest sequence as the OTU-representative sequence. These sequences were subsequently aligned with the Python Nearest Alignment Space Termination (PyNAST) algorithm (Caporaso et al., 2010a, 2010b). Chimeras and singletons were detected and removed with ChimeraSlayer (Haas et al., 2011) as implemented in QIIME. Finally, taxonomic assignment were carried out by using SILVA 128 database (http://www.arb-silva.de/). To confirm the correct taxonomic assignation of *C*Xc 454 ribotypes, we aligned them with other *16S rRNA* sequences, including bona fide *C*Xc sequences, and reconstructed a Neighbor-joining (NJ) tree using MEGA 6 (Tamura