Study of the interspecific variability of the clam pathogen *Vibrio tapetis* using Multilocus Sequence Analysis (MLSA) and DNA-DNA hybridization (DDH).

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Introduction

Vibrio tapetis is the causative agent of an epizootic infection described in adult clams called Brown Ring Disease (Borrego *et al*., 1996). The main sign characterizing the disease is a brown conchiolin deposit on the inner surface of the valves. Typically located between the pallial line and the edge of the shell, this deposit perturbs the calcification process (Paillard *et al*, 1994, Paillard, 2004) causing severe deformations of the clam's shell and subsequently the death of the animal.

First studies indicated that strains of this pathogen constituted a homogenous group on the basis of their antigenic, phenotypic and genotypic characteristics. However, as new strains were isolated from different hosts, including different mollusk and fish species, some variability was demonstrated.

The genetic variability of this bacterial was studied using different typing methods based on both restriction and PCR-based protocols. Restriction fragment length polymorphism (RFLP) and pulse field gel electrophoresis (PFGE) have been used in the study of this pathogen. The last one has been considered as the best strategy for typing bacteria, but the process is laborious, time consuming, and technically demanding, which limits its routinely use (Romalde 2005).

Rep-PCR is a group of techniques that generates DNA fingerprints which can be utilized for the discrimination of bacterial species and/or strains (Versalovic *et al.* 1991, 1994). These methods involve the application of oligonucleotide primers based on families of short, highly conserved extragenic repetitive sequences, including the repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC) sequences (Stern *et al.*1984, Hulton *et al.*1991). Randomly amplified polymorphic DNA analysis (RAPD) is based on the use of a single short random sequence primer to obtain a specific bands pattern species or strain specific (Welsh & McClelland 1990, Williams *et al.*1990).

All this techniques lead to the description of three main subgroups in this species that correlate with the host type (Castro *et al.*1996, 1997; Le Chevalier *et al.*2003; Rodríguez *et al.*2006; Romalde *et al.*2002).

MLSA (Multilocus Sequence Analysis) is a typing method based on the concatenation of the sequence of several housekeeping genes. This technique has been successfully used on epidemiological assays and, more recently in species delineation and in the study of relationships between bacterial groups. This approach has been demonstrated to be very useful and accurate for vibrios, being very congruent with results of DNA-DNA hybridization (Thompson *et al.*2009).

Material and Methods

A total of 28 strain were employed in the study including 16 isolates from manila Clam (*Ruditapes philippinarum*), being two of them isolated from seed, 2 from carpet-shell clam (*R. decussatus*), 1 from aurora venus clam (*Venerupis pullastra*), 1 from cockle (Cerastoderma edule), 4 from wedge sole (*Dicologoglossa cuneata*) 3 from shi drum (*Umbrina cirrosa*) and 1 from halibut (*Hippoglossus hippoglossus*).

Genomic DNA extraction and amplification of the 16S rRNA gene was performed as previously described (Beaz *et al.,* 2009). Sequences for four protein-coding housekeeping genes *rpoA* (α subunit of RNA polymerase), *recA* (recombinase A), *pyrH* (ulidyl monophospate kinase) and *atpA* (α subuntil of atpase) were performed according to Thompson *et al.* (2004, 2007)and Thompson *et al*. 2005. Sequencing reactions were performed with the GenomeLab DTCS-

Quick Start Kit (Beckman Coulter). Sequence data analysis was performed with DNAstar Seqman program (Lasergene, USA). Sequence nucleotide alignments were revised visually to identify positions with uncertain alignments, mainly at both ends of the sequences, to be corrected or omitted for further analyses.Phylogenetic analysis was performed using the program Mega version 4.0.1 (Tamura et al., 2007). Phylogenetic trees were constructed by neighbour-joining (NJ) (Saitou & Nei, 1987), distance matrices were calculated using Kimura´s two parameter correction, and maximum parsimony (MP) using a heuristic approach. Bootstrap analyses were performed using 1000 replications for both analyses.
DNA–DNA hybridization (DDH) experiments, including reciprocal analysis, were done in duplicate by the hydroxyapatite/microtitre plate method (Ziemke et al., 1998) with a hybridization temperature (Tm) of 60 ºC.

Results
Phylogenetic reconstruction based on the concatenation of four genes (Fig.1) showed three groups, each one containing one of the representative strains of the three groups previously described for the species. The first group, represented by the type strain CECT 4600^T contains all manila clam isolates as well as cockle, soft carpet-shell clam, wedge sole and two corb isolates. The second group contains the carpet shell clam isolates and one corb strain. The third group is formed by halibut and manila clam seed isolates. The similarity of the concatenated genes was in all cases more than 98%.
Preliminary resulys on DDH supported the existence of these three groups, although further studies are needed including more isolates to definitivelyclarify the existence of genomovars or even subspecies within *V. tapetis*.

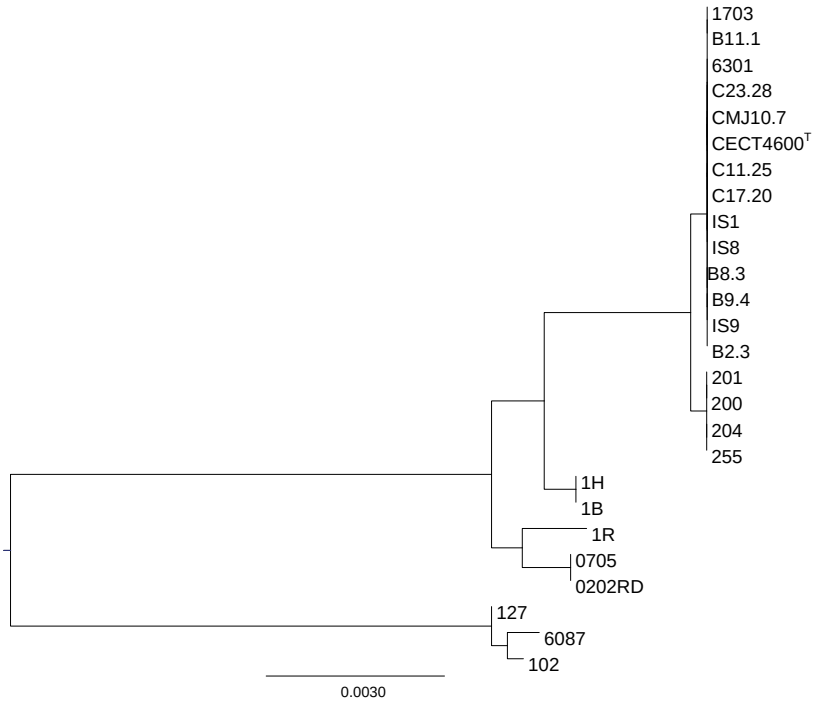

Fig 1: Phylogenetic reconstructions based on concatenated 16S rRNA, *recA*, *pyrH*, *atpA* and *rpoA* gene sequences. Analyses were done using Maximum Likelihood method.

Discussion
As new isolates are discovered the complexity of this pathogen species is increasing. The MLSA assay confirm the groups corresponding to manila and carpet shell clam but fish isolates

seem to be in an incertae sedis position. Thus, wedge sole isolates grouped with manila clam isolates, as it was demonstrated by ERIC-PCR (López et al. 2010). Seed isolates are placed with halibut isolate, the three isolates share common biochemical features different for the rest of the species. In the case of corb isolates, all of them are more related with clam isolates than with the previously described fish group. Two of them are comprised in carpet shell clam group while the other two formed a branch by themselves.

In summary, the concatenation of five genes has demonstrated to be a very good tool for the study of the variability of *Vibrio tapetis*, confirming the diversity already described and suggesting the existence of a new genetic group in this species.

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