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Short Communication

A strain of piscine myocarditis virus infecting Atlantic argentine, *Argentina silus* (Ascanius)

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Cardiomyopathy syndrome (CMS) in Atlantic salmon, *Salmo salar* L., is an inflammatory disease of the heart, generally affecting farmed fish 12–15 months after transfer to sea water (Ferguson, Poppe & Speare 1990). CMS causes significant losses in the fish farming industry, and CMS-like clinical signs have also been described in wild fish (Poppe & Seierstad 2003). The newly described piscine myocarditis virus (PMCV) is believed to be the causative agent (Lovoll *et al.* 2010; Haugland *et al.* 2011). Until recently, the virus had been reported exclusively in salmon sampled in association with CMS outbreaks, but our group has also detected PMCV in approximately 6.6% of Atlantic argentine, *Argentina silus* (Ascanius), collected along the Norwegian coastline (Böckerman *et al.* 2011). By generating sequence data, performing broad-range PCR and quantifying PMCV in different *A. silus* tissue types, we have investigated the possible role this strain of the virus has in the development of CMS.

Using the previously published real-time PCR assay for PMCV detection target locus as a starting point (Lovoll *et al.* 2010), a partial sequence of the RNA-dependent RNA polymerase gene (*RdRP*) in PMCV from *A. silus* was generated by primer walking. Multiple overlapping amplicons were generated, and a consensus sequence was con-

structed (GenBank accession number JN624781). The 376 amino acid protein sequence was virtually identical to the data reported from PMCV in Atlantic salmon [97% identity, 99% positive and no gaps using protein BLASTP (protein–protein BLAST)], but on a nucleotide level the sequences from the two virus isolates were quite different with just 86% identity across the corresponding 1128 nucleotides.

What appeared to be the most conserved core of the RdRP protein sequence was aligned with other members of Totiviridae using the ClustalW multiple alignment algorithm (Thompson, Higgins & Gibson 1994). The alignment was subsequently adjusted manually to ensure that known functional domains such as the GDD polymerase motif were correctly lined up. A phylogenetic analysis was performed using maximum likelihood and the program GARLI (version 0.96b8; http://www.nescent.org/wg_garli/Main_Page) with default parameters and five search replicates (genthreshfortopoterm = 10000). A bootstrap analysis was performed using the same parameters (100 pseudoreplicates), and a majority rule consensus tree was plotted using the program Consense (version 3.69, part of the PHYLIP package; <http://evolution.gs.washington.edu/phylip.html>).

The analysis showed the two PMCV strains as distinct members of a highly supported, paraphyletic group that includes all totiviruses infecting higher order, multicellular organisms in addition to giardaviruses (Fig. 1). The newly suggested genus *Artivirus* infecting insects such as *Armigeres subalbatus* and *Drosophila melanogaster* (Zhai *et al.* 2010) is also included in this clade. An evolutionary link between all of these viruses is interesting, and further studies

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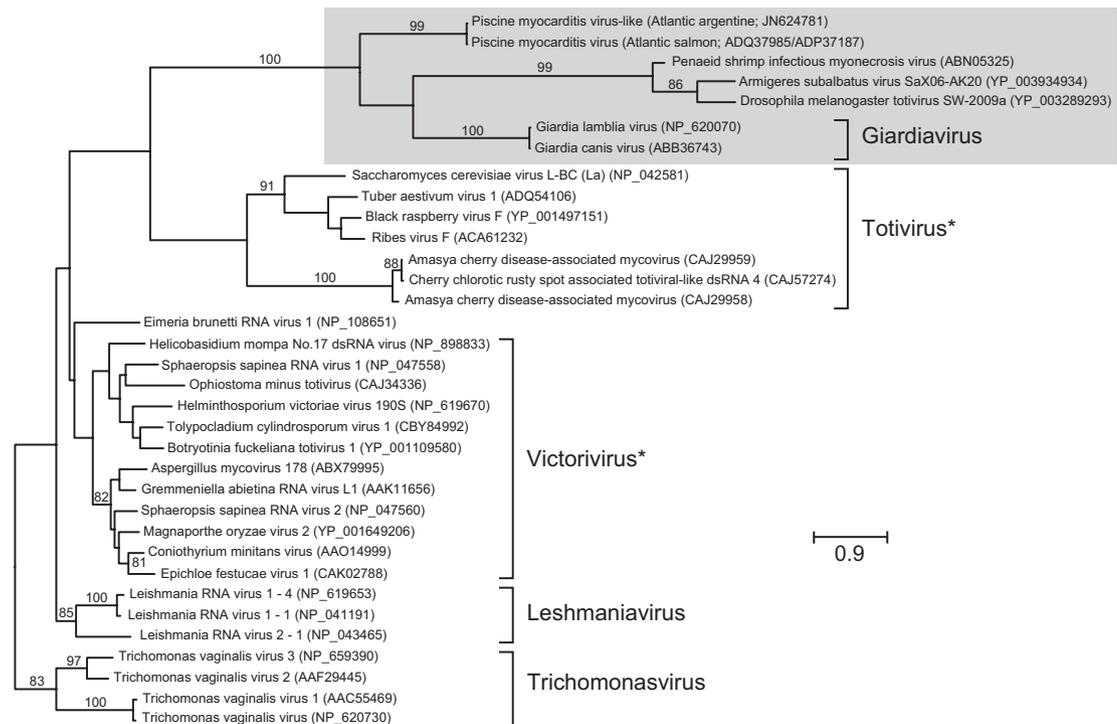


Figure 1 Phylogenetic analysis of totiviruses using maximum likelihood and amino acid sequences (partial *RdRP*). Bootstrap values above 80% have been indicated. *Genus includes certain sequences listed as 'unclassified' in the NCBI Taxonomy database.

on this group will reveal whether there are any markers of a switch in host specificity defining this cluster.

When looking at the nucleotide sequence alignment, the differences between the two PMCV sequences were scattered throughout. There were only a handful of conserved regions, and these were used for primer design. By targeting conserved regions, we wanted to develop a method for broad-range screening for PMCV-like viruses. A previously described set of 342 RNA pools (1501 individuals) representing 32 marine fish species (Böckerman *et al.* 2011) was screened using two conserved primers spanning a 711 nucleotide region of the genome (PMCVcons1F: ATTATGTAGG GATAAATGGGC and PMCVcons1R: GAAG AAATTAACGCTTTAGG). Primer selectivity was ensured using the program UniquePrimer (Nakken *et al.* 2009). PCR was carried out using the Qiagen One-Step RT-PCR Kit, according to the manufacturer's recommendations and the following PCR cycle: 30 min at 50 °C (reverse transcription), 15 min at 94 °C (reverse transcriptase inactivation and PCR polymerase activation), 45 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/1 min and a final

elongation step (3 min at 72 °C). PCR products were checked using agarose gels.

For viral quantification in different *A. silus* tissue types, we developed a method where SYBR Green I Nucleic Acid Gel Stain (Molecular Probes, Inc.) was added directly to the Qiagen One-Step RT-PCR Kit mastermix at 1.5× final concentration. The same primers as for the conventional PCR were used. Using a standard amount of input RNA (100 ng), viral RNA loads of heart, spleen, kidney, brain, gills and gonads from a set of five fish that were positive with the PCR screening assay were assessed. In addition to the real-time fluorescence curves, dissociation curves and agarose gels were also used to ensure the accuracy of these real-time PCR runs.

Polymerase chain reaction screening using the conserved primers gave results that were virtually identical to the results we obtained previously using the PMCV real-time PCR (Böckerman *et al.* 2011). We believe that this shows that even though there are two different strains of the virus in species as distantly related as *S. salar* and *A. silus*, this group of viruses does not appear to be common in marine fish. Gonad tissue was only available from two samples, and both were negative. All other tissues

were positive and contained variable amounts of viral RNA (ranked from highest to lowest load): kidney, spleen, gill, heart and brain. Relatively high amounts of PMCV RNA in kidney, spleen and heart tissue have also been observed in *S. salar* suffering from CMS (Dr S. M. Jørgensen, personal communication), so our results do not exclude the possibility of PMCV also being a potentially harmful virus in *A. silus*.

The distinct genotype of PMCV found in *A. silus* vs. *S. salar* makes it less likely that this virus reservoir is important for the development of CMS in farmed salmon. The two sequences reported thus far from PMCV (GenBank accession numbers HQ401057 and HQ339954) have come from different sources, and yet they are 100% identical, indicating that the virus relevant for CMS in farmed fish in Norway is relatively homogenous. It is thus unlikely that the genetic differences we have observed merely reflect population genetics. We have previously suggested that one possible route of transmission could be *A. silus* used in fish feed production, but PCR screening of RNA extracted from a selection of fish pellets has thus far not given any positive results (data not shown).

Future efforts in identifying virus reservoirs causing PMCV infections in salmon should include testing of a broad range of biological materials and organisms that are in the proximity of fish farms, including protists, jellyfish and sediments. Representative crustacean species should also be tested as PMCV is closely related to the penaeid shrimp infectious myonecrosis virus that affects muscle tissue in shrimp (Fig. 1).

When aligning primer and probe sequences from our original PMCV-specific assay with the sequence derived from *A. silus*, we noticed that there was one mismatch in the forward primer, two mismatches in the MGB probe and three mismatches in the reverse primer. MGB probes are considered among the most specific PCR-based tools available, but our assay gave a positive signal when testing *A. silus* material (Böckerman *et al.* 2011). This underscores the importance of PCR-protocol optimization and extensive sequencing of primer/probe regions when working with molecular detection assays.

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