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Molecular detection of betanodavirus from the farmed fish, *Platax orbicularis* (Forsskal) (Ephippidae), in French Polynesia

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In French Polynesia, the «service de la pêche» (SPE, «Fisheries Office», a local government agency) has initiated together with the Ifremer (the French research institute for exploitation of the sea) a research project on the domestication of a native lagoon fish species, the orbicular batfish *Platax orbicularis*. The original broodstock consisted of wild caught individuals from Tahiti and Bora Bora islands (French Polynesia) reared in the hatchery with other tanks of *Polydactylus sexfilis* and *Lates calcarifer* breeders. However, this breeding program rapidly faced mortalities. Diseased larvae exhibited prominent clinical signs such as anorexia, darkening of the body, emaciation of gills and spiral swimming behavior. These clinical signs were accompanied by mass mortality.

Limited information was available in the literature regarding this species and no diseases of *P. orbicularis* had been reported. Thus, infected larvae (N=24) of *P. orbicularis* were sampled from two batches of larvae reared in the Ifremer-SPE hatchery (Tahiti, French Polynesia). Samples were taken from 20 days-old to 23 days-old larvae presenting mortality rates up to 88%. For the histological studies, whole larvae were fixed in buffered Davidson fixative and processed using standard procedures for paraffin histology and stained with haematoxylin and eosin (serial sections from the same tissues were also retained and placed onto 2% silane pre-treated slides for *in situ* hybridization). Vacuolation and necrosis were evidenced in nerve cells of brain, spinal cord and retina on 16 (66%) of the affected fish (figure 1B), unlike in healthy fishes (figure 1A). In the brain, diffuse or multifocal regions of vacuoles produced a spongiform appearance. These clinical observations strongly suggested a betanodavirus infection. Indeed, such a typical nerve necrosis manifestation in the brain tissue and the retinal layer of larvae has already been reported in VNN infected *L. calcarifer*, *Dicentrarchus labrax*, *Epinephelus aeneus* or *Mugil cephalus* (Renault, Haffner, Breuil & Bonami 1991) (Ucko, Colorni & Diamant 2004) (Azad, Shekhar, Thirunavukkarasu & Jithendran 2006).

The betanodavirus (member of the *Nodaviridae* family) is the aetiological agent of the viral nervous necrosis (VNN) disease. This disease, also known as viral encephalopathy and retinopathy (VER), is an infection characterized by necrosis of the central nervous system (Ito, Okinaka, Mori, Sugaya, Nishioka, Oka & Nakai 2008) (Bovo, Nishizawa, Maltese, Borghesan, Mutinelli, Montesi & De Mas 1999). In the last few years, VNN has become a serious threat to marine fish farming, causing massive mortalities in larvae and juveniles in more than 30 fish species around the world (Gomez, Baeck, Kim, Choresca & Park 2008) (Munday, Kwang & Moody 2002). The spread of VNN among populations of cultured marine fishes has been attributed to either vertical or horizontal transmission (Gomez *et al.* 2008). Anyway, VNN worldwide distribution has not yet been fully investigated and the fact that the betanodavirus remains undetected in some regions may reflect a lack of information rather than the result of a true absence (Ucko *et al.* 2004). In French Polynesia, VNN was first detected in Tahiti in 1989 affecting larvae of cultured *Lates calcarifer* (Renault *et al.* 1991).

Based on our previous observations, *in situ* hybridization was performed to confirm the betanodavirus infection of *P. orbicularis*. Probes were PCR-labelled using DIG-dUTP incorporation. PCR was performed with cDNA from *L. calcarifer* betanodavirus from the French Polynesian strain as template using primers F1 (5'-ACACTGGAGTTTGAAATTCA) and R2 (5'-GGCAGCAGGATTTGACGGGGCTGC). These primers were designed after the alignment of available betanodavirus coat protein gene sequences based on the work of Dalla Valle *et al.* (Valle, Negrisolo, Patarnello, Zanella, Maltese, Bovo & Colombo 2001). Paraffin tissue sections on aminoalkylsilane-coated slides were dewaxed and rehydrated. Proteinase K (100 μg/ml) in Tris-EDTA buffer was applied for 30 min at 37°C, and sections were dehydrated by immersion in ethanol series and air dried. Sections were incubated with 100 μl of hybridization buffer (4X SSC, 50% formamide, 1X Denhardt's solution, 250 μg/ml yeast tRNA, 10% dextran sulfate) containing 20 ng of digoxigenin-labeled probe. Sections were

covered with *in situ* plastic coverslips and placed on a heating block at 95°C for 5 min, then cooled on ice and left overnight at 42°C in a humid chamber. Sections were washed and detection was done with the DIG nucleic acid detection kit according to manufacturer's instructions (Roche - Boehringer Mannheim). The probe strongly reacted with putative infected tissues without any background hybridization on *P. orbicularis* tissues. Virus aggregates were localized throughout the different ocular layers, brain tissue and spinal cord (figures 2A and B). Negative control (safe larvae) did not show any labelling (figure 2C). Results were checked by conventional topographic staining of the histological sections to confirm the identity of the labelled cells. Similar tissue localization was observed by electronic microscopy (TEM) on the Asian seabass *L. calcarifer*, where virus particles were detected in the cytoplasm of nerve cells of the brain, spinal cord and retina (Azad *et al.* 2006). These results are consistent with the fact that betanodaviruses are believed to spread along nerves (Nguyen, Nakai & Muroga 1996).

Betanodaviruses are non-enveloped spherical viruses with a genome consisting in two segments of positive-sense single stranded RNAs. The first segment, RNA1 (3.1 kb), encodes the viral replicase (Tan, Huang, Chang, Ngoh, Munday, Chen & Kwang 2001), whereas the second, RNA2 (1.4 kb), encodes the coat protein (Delsert, Morin & Comps 1997). In addition, RNA3, a subgenomic transcript of RNA1, encodes the B2 protein, which has a suppressor function in post-transcriptional gene silencing (Iwamoto, Mise, Takeda, Okinaka, Mori, Arimoto, Okuno & Nakai 2005). Based on partial nucleotide sequence of the coat protein gene, betanodaviruses have been classified into four genotypes: the striped jack nervous necrosis virus (SJNNV), the barfin flounder nervous necrosis virus (BFNNV), the tiger puffer nervous necrosis virus (TPNNV) and the red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa, Furuhashi, Nagai, Nakai & Muroga 1997, Valle *et al.* 2001). Partial *P. orbicularis* betanodavirus coat protein specific gene (618 bp) was characterized using the same primers (F1 and R2) used for the *in situ* hybridization. Total RNA was extracted from

fish tissue using Trizol reagent according to the manufacturer instructions (Invitrogen™). cDNAs were amplified using 1 µg of total RNA according to the manufacturers protocol (Promega). PCR amplification was performed using the same primers, F1 and R2, at a concentration of 2µM. PCR products were gel purified using the Geneclean® II extraction kit (Q-BIOgene, France) and cloned using TOPO TA (Invitrogen™) with the pCR 2.1 TOPO ® vector. Plasmids were transformed into TOP10 *E. coli* cells, and plasmid was purified using Wizard™ SV miniprep DNA purification kit (Promega). Plasmids sequencing was performed at Cogenics Genome Express (Meylan, France). Two nucleotidic sequences, presenting 99.7% identity with each other, were obtained from betanodaviruses isolated from *P. orbicularis*. These two isolates showed high nucleotidic identity with the 618 bp nucleotidic sequence of the RNA2 coat protein (between 94.3% and 95.6%) of viruses from the RGNNV type group. A lower nucleotidic identity (between 77% and 82.4%) was observed with sequences from the three other groups. Additionally, phylogenetic trees clearly confirmed that the betanodavirus isolated from the tahitian *P. orbicularis* clustered within the RGNNV type group (figure 3). These results also confirmed that betanodaviruses, having low host-fish species specificity, could occur in a large scope of marine fishes.

These data also confirmed the betanodavirus infection suspicion of *P. orbicularis* in the Ifremer-SPE hatchery (Tahiti, French Polynesia). Besides, VNN diseases were previously diagnosed in Tahiti affecting cultured fish larvae of *L. calcarifer* (Renault *et al.* 1991) and cultured juveniles of wild-caught post larvae of *Acanthurus triostegus* and *Apogon exostgma* (Thiery, Cozien, de Boisseson, Kerbart-Boscher & Nevarez 2004). In our program, the original broodstock of *P. orbicularis* consisted of wild caught individuals from Tahiti and Bora Bora islands (French Polynesia) reared in the hatchery with other tanks of *P. sexfilis* and *L. calcarifer* breeders. The 618 bp nucleotidic sequences from betanodaviruses isolated from *P. orbicularis* and *L. calcarifer* presents high level of identity (99.2%). Even if the possibility

that theses subtypes could belong to the same virus strain, it is not possible with our set of data to rule out the origin of the Tahitian nodavirus.

In the present study, we report the first histological and molecular detection of a betanodavirus in the fish *P. orbicularis*. It concerns larvae reared in the Ifremer-SPE hatchery (Tahiti, French Polynesia). Hence, a breeding strategy based on health prevention has been developed. The main aim was to prevent pathogen introduction in our experimental hatchery. As the spread of VNN disease among cultured fish population could be attributed to either vertical or horizontal transmissions, it is therefore important to detect virus carriers to steer clear of its spread. To this end and based on the results obtained in this study, we have developed a screening method by RT-PCR to detect betanodaviruses in new progenitors or larvae. Thus, this program will allow us to propose bio secured process in hatcheries in order to develop a sustainable aquaculture with respect to environment and animal welfare.

Figure legends

Figure 1: Histopathological observations of (A) healthy and (B) VNN affected *P. orbcularis* larvae at 20 days post-hatch (light microscopy, hemalun-eosine staining (scale bars 40mm). Arrows indicate vacuolation in the retina (r) and the brain (b). Abbreviation (sc) corresponds to the spinal cord.

Figure 2: *In situ* hybridization on paraffin sections of VNN infected *P. orbicularis* tissues using betanodavirus specific probe. A. Larvae show labelling in the brain (b) and the spinal cord (sc). (scale bar 50mm). **B.** Arrows indicate prominent labelling of ganglionic cell layers in the eye. (scale bar 14mm). **C.** Negative control: no labelling is observed in non infected *P. orbicularis* eye. (scale bar 14mm). Dark staining indicated by arrow heads corresponds to melanin in the three pictures.

Figure 3: Phylogenetic analyses of betanodavirus isolates from *P. orbicularis*. Unrooted phylogenetic tree based on the partial nucleotidic sequence of the RNA2 coat protein was constructed using the the neighbour-joining method of the MEGA package (http://www.megasoftware.net/). Betanodaviruses from the batfish *P. orbicularis* (PNODA2 [GQ233029] and PNODA7 [GQ233030]) were analyzed with related sequences available in GenBank. European seabass *Dicentrarchus labrax* (AF175520); Japanese flounder *Paralichthys olivaceus* (D38527); Barramundi *Lates calcarifer* (EF591372); Cobia *Rachycentron canadum* **(**AY140795); Redspotted grouper *Epinephelus akaara* (D38636); Guppy *Poecilia reticulata* (AF499774); Rock porgy *Oplegnatus punctatus* (AF175520); Striped Jack *Pseudocaranx dentex* (D30814); Tiger puffer *Takifugu rubripes* (D38637); Barfin flounder *Verasper moseri* (D38635); Atlantic halibut *Hippoglossus hippoglossus* (AF160473); European seabass *Dicentrarchus labrax* (AJ698094); Atlantic cod *Gadus morhua* (AF445800). Bootstrap values are indicated as percentages of 1000 replicates.

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Figure 1

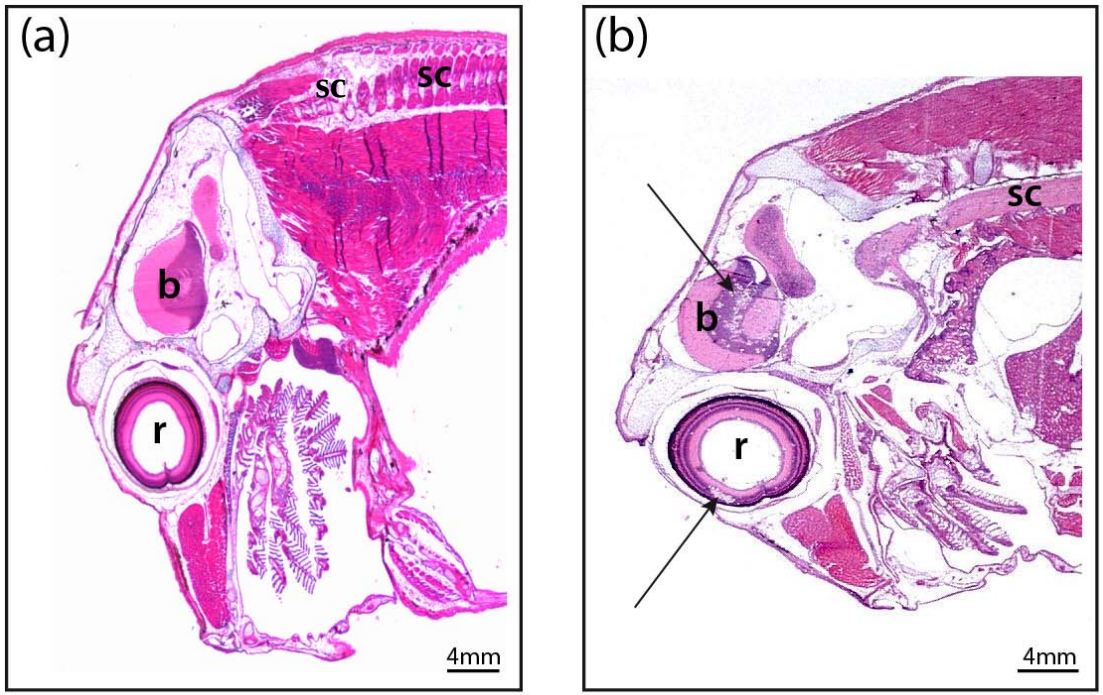


Figure 2

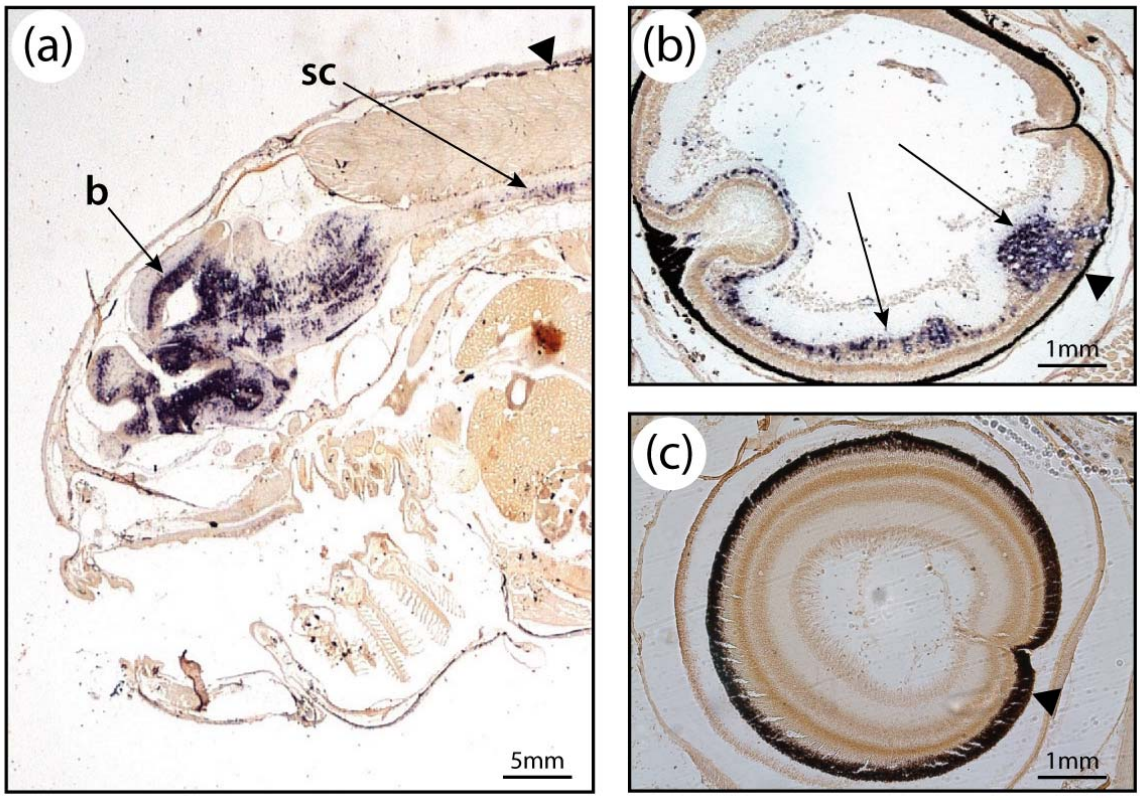
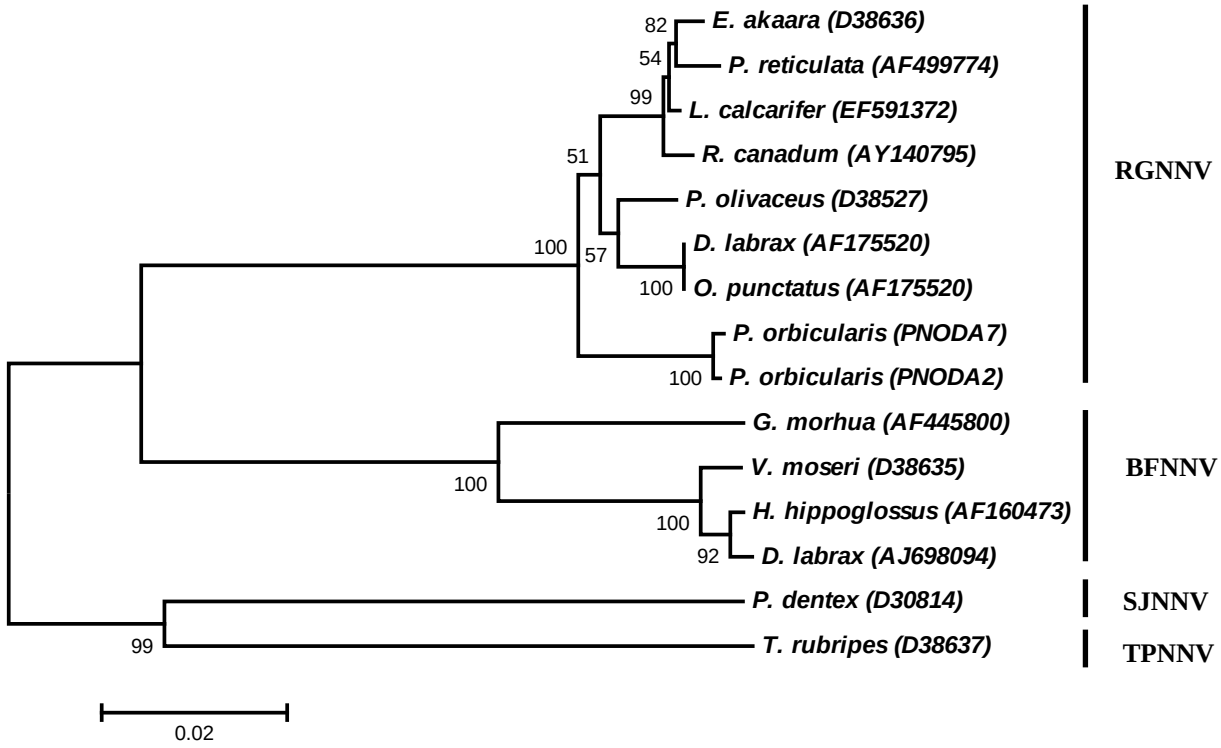


Figure 3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L. calcarifer (EF591372)
R. canadum (AY140795)
P. olivaceus (D38527)
D. labrax (AF175520)
O. punctatus (AF175520)
P. orbicularis (PNODA7)
P. orbicularis (PNODA2)
G. morhua (AF445800)
V. moseri (D38635)
H. hippoglossus (AF160473)
D. labrax (AJ698094)
P. dentex (D30814)
T. rubripes (D38637)
RGNNV
BFNNV
SJNNV
TPNNV
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