Isolation of betanodavirus from farmed turbot *Psetta maxima* showing no signs of viral encephalopathy and retinopathy

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25 Abstract. The isolation of a betanodavirus strain from juvenile turbot showing
26 unspecific symptoms and very low mortality is reported. The presence of an IPNV-type
27 virus was also revealed in the fish tissues, but only after nested-PCR**,** suggesting a very
28 low viral load. In addition, different *Vibrio* species were isolated from some of the
29 individuals. Both viral RNAs were sequenced and phylogenetic analysis indicated that
30 the strain showed 99% identity with the RGNNV genotype. Experimental infections
31 (via immersion and intraperitoneal route) were conducted in order to determine the
32 susceptibility of turbot juveniles (2 and 5g) to the NNV isolate at 15 and 18 ºC (range of
33 turbot rearing temperatures in our area). The results obtained indicated that although the
34 viral isolate was able to replicate in the turbot tissues, it did not induce clinical disease
35 in this fish species. These findings suggest that the existence of a reservoir of NNV-
36 RGNNV type in wild fish in the area represents a low risk for the turbot farming
37 industry.

38 Key words: Turbot, betanodavirus, RGNNV, susceptibility, clinical disease, viral
39 replication

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1. Introduction

Viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) is a serious neuropathological condition causing important mortalities in the larvae and juveniles of an increasing number of fish species around the world. The etiological agents of the disease are viruses belonging to the genus *Betanodavirus* (family *Nodaviridae*). Betanodaviruses are non-enveloped virions with an icosahedral symmetry, about 25 nm in diameter and a viral genome consisting of two single-stranded (ssRNA) segments, the RNA1 and RNA2. The RNA1 (3.1 Kb) encodes the viral replicase (Nagai and Nishizawa 1999; Tan et al., 2001), whereas the RNA2 (1.4 Kb) encodes the coat protein (Delsert et al., 1997: Nishzawa et al., 1994). In addition, a subgenomic RNA, termed RNA3, is transcribed from the 3' end of RNA1 (Sommerset and Nerland 2004; Iwamoto et al., 2005). On the basis of the comparison of a variable region of the coat protein gene, the betanodavirus are classified in four genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), red-spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) (Nishizawa et al., 1995). A fifth genotype, namely the turbot nervous necrosis virus (TNV) type was proposed after sequencing the PCR product obtained from turbot affected by a natural outbreak of VER in Norway (Johansen et al., 2004), but the virus was not isolated. Although no other reports have been published on VER infections in turbot so far, one of the first descriptions of nodavirus in fish, reported as encephalomyelitis, was made in this species (Bloch et al., 1991) as indicated by Munday et al. (2002) in their review. In addition, experimental infections have shown that this species is susceptible to nodavirus belonging to SJNNV and BFNNV genotypes (Husgard et al., 2001; Sommerset et al., 2003).

In recent years the detection of betanodavirus in apparently healthy wild fish (including turbot, but mainly flathead grey mullet, *Mugil cephalus*, blackspot seabream *Pagellus bogaraveo,* and common seabream, *Diplodus vulgaris*) has increased notably in our area, as reported by the Spanish *Junta Nacional Asesora de Cultivos Marinos* (JACUMAR) (http://www.magrama.gob.es/app/jacumar/planes_nacionales/Documentos/100_IF_ GESAC_ EPIDEMIOLOGIA_Anexo_I_GALICIA.pdf)

In the present study we report the isolation of a nodavirus strain from juvenile farmed turbot showing low mortalities and unspecific symptoms. Both viral RNAs were sequenced and phylogenetic analysis indicated that the strain showed 99% identity with RGNNV genotype. Experimental infections were performed in order to evaluate the susceptibility of turbot juveniles to this isolate.

2. Materials and methods

2.1. Fish analysis.

Fourteen turbot (5 g average weight) showing swollen abdomen and mild levels of mortality (approximately 0.1% which lasted a month) were received in the facilities of the Instituto de Acuicultura (Universidad de Santiago de Compostela) at the end of December 2010 and subjected to virological analysis. Fish were sacrificed with a MS-222 overdose, necropsied under aseptic conditions and examined for internal lesions.

Spleen, kidney, and brain were aseptically collected from each fish. Brain samples were pooled and used for betanodavirus isolation, whereas pools of spleen and kidney were tested for the presence of viral haemorrhagic septicaemia (VHSV), infectious haematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). Tissue samples (brain and kidney/spleen) were mixed (1:10) with Earle's balanced salt solution (Hyclone Laboratories Inc.) supplemented with antibiotics (1000 UI ml^{-1}

93 penicillin, 1000 µg ml^{-1} streptomycin, 500 µg ml^{-1} gentamycin and 10 µg ml^{-1}
94 amphotericin B) and homogenized. After centrifugation of the homogenates at 2000 g
95 for 20 min, the supernatants were transferred to new tubes, and incubated for 4 hours at
96 15 ºC. Afterwards, the supernatans where inoculated (diluted at 10^{-1} and 10^{-2}) in
97 duplicate onto 48-well plates of semiconfluent monolayers (around 80% confluence) of
98 chinook salmon embryo (CHSE-214), bluegill fry (BF-2) and epithelioma papillosum
99 cyprini (EPC), which where used for kidney/spleen samples, or E-11 (a clone of the cell
100 line striped snakehead SSN-1), which was used for brain samples. CHSE-214, BF-2 and
101 EPC were grown in Eagle's minimum essential medium (EMEM, Hyclone Laboratories
102 Inc.) at 15, 20 and 20ºC, respectively, and E-11 was grown in L-15 (Gibco) at 25ºC.
103 Growth media were supplemented with 10% foetal calf serum (FCS, BioWhittaker),
104 100 I.U. ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. When monolayers were
105 semiconfluent, the medium was substituted by fresh medium with 0 or 2% FCS for viral
106 infection. All infected monolayers were incubated at 15ºC, and examined daily for the
107 presence of cytopathic effect (CPE). After 15 d, positive and negative samples (cultures
108 showing CPE or no-CPE, respectively) were subcultured by inoculating 0.1 ml of the
109 scraped cell suspension onto new cultures. Subcultures were terminated after 15 d of
110 incubation. Non-infected cells were used as controls.
111

112 *RT-PCR and nested PCR detection*

113 Total RNA was extracted from aliquots of the tissue samples using RNeasy Mini kit
114 (Qiagen) following the manufacturer´s instructions. Primer pairs used for RT-PCR were
115 Heppel F/R for IPNV (Heppel et al., 1992), cm3a and cm3b for VHSV (López-Vázquez
116 et al., 2006), and F2-R3 (Nishizawa et al., 1994) for nodavirus. Complementary DNA
117 (cDNA) synthesis was performed by mixing the viral RNA with 2.5 ng $µl^{-1}$ of random

118 primers (Promega) heating at 95ºC for 5 min and incubating at 4ºC for at least 1 min.
119 Then a reverse transcription mixture containing Superscript III RT (Invitrogen) was
120 added and incubated at 25° C for 10 min. The RT reaction was performed at 50° C for
121 50 min, followed by a 5 min/85 ºC RT enzyme inactivation. The PCR reaction was
122 performed using 4 µl of cDNA, 1.25 U of GoTaq® Flexi DNA polymerase (Promega)
123 and 0.5 µM of the specific primer set. Following an initial 4 min denaturation step at
124 94° C, the mixture was subjected to 40 cycles of amplification (30 s at 94°C, 30 s at
125 58°C, and 30 s at 72°C) with a final extension of 10 min at 72°C. The PCR products
126 were analysed by electrophoresis on a 1.5% SeaKem® LE agarose gel (FMC
127 Bioproducts)
128 The nested RT-PCR was performed using 3 µl from the RT-PCR reaction and the
129 specific primers Heppel introF (5'-AAAGGCATGGGGCTGGAGAG-3')/HeppelR for
130 IPNV *cm3aintro* and *cm3bintro* for VHSV (López-Vázquez et al., 2006) and F21-R31
131 for nodavirus (Olveira et al., 2008), using the same protocol as described above.

132 ***2.2.*** *Sequencing and phylogenetic analysis*

133 The sequence of the coding regions of both RNA segments from the betanodavirus
134 isolate was determined using the primer walking approach. A panel of 9 sets of primers
135 (6 for RNA1 and 3 for RNA2) corresponding to overlapping regions of the
136 betanodavirus genome (Olveira et al., 2009) were used.

137 Automated sequencing was performed using a CEQ^{TM} 8000 Genetic Analysis
138 System (Beckman Coulter). Sequences of both segments were confirmed at least twice
139 by sequencing upstream and downstream and, to solve any inconsistences a second set
140 of sequencing was performed. The sequences were edited using DNASTAR
141 Lasergene® v.7.1 SeqMan II and EditSeq (DNASTAR). For comparative purposes,

142 nucleotide sequences of reference strains of betanodavirus deposited in the GenBank
143 were used (Table 1). All sequences were subjected to multiple sequence alignment
144 using the DNASTAR Lasergene v.7.1 MegAlign program (DNASTAR). Following
145 alignment, trees were constructed by Bayesian inference of phylogeny using BEAST
146 1.7.5 (Bayesian MCMC analysis of molecular sequences) (Drummond et al., 2012) and
147 employing the GTR+G (RNA1) and HYK+G (RNA2) models. Three Markov chains
148 were run for 10,000,000 generations and Bayesian posterior probabilities (PP) were
149 obtained from the 50% majority rule consensus of trees sampled every 100 generations
150 after removing the 50,000 first generations. Sequences of an alphanodavirus – BBV
151 [black beetle virus, accession no. NC_001411 (RNA1) and NC_002037 (RNA2)] –
152 were used as an outgroup to produce rooted trees.

153 ***2.3.*** *Experimental infection*

154 ***2.3.1.*** *Trial 1.*

155 Turbot (average weight 5 g) were obtained from a commercial fish farm. On arrival at
156 the facilities of the University aquarium fish were divided into two groups (G1 and G2)
157 and gradually acclimated to the desired temperature (15 or 18º C, respectively) for 15
158 days. Water temperature was then maintained during the experimental infection. Prior to
159 challenge, 20 fish from each group were examined for the presence of NNV, IPNV,
160 IHNV and VHSV by inoculation in cell culture and RT-PCR

161 Turbot were stocked at a density of 40 fish per tank in 100 l aquaria and exposed to
162 virus at a concentration of 10^5 $TCID_{50}$ ml^{-1} in a total volume of 3l for 3 h. As negative
163 controls, two tanks (one per temperature) containing ten fish each were also set up using
164 MEM with no virus. The experiment was terminated after 30 days. Fish were monitored
165 daily for signs of disease and mortality. Brain and eye samples from dead and surviving
166 fish were sampled and stored at -80ºC for virological examination. Control fish were

tested for viral presence (IPNV, IHNV, VHSV and NNV) at the end of the trial as described above.

2.3.2. Trial 2.-

Turbot were obtained from the same farm as in trial 1, but in this experiment two different sizes of fish were used (2 and 5 g). Fish were checked for viral presence and acclimated to temperature as described above.

Turbot were held in 100 l aquaria and stocked at a density of 60 fish per tank in six tanks, named G1A, G1B, and $G1B_{ip}$, G2A, G2B and $G2B_{ip}$. Tanks from group 1 (G1) were maintained at 15º C and tanks from G2 at 18º C. Small fish (2g) were distributed in tanks A and bigger fish (5g) in tanks B.

Infection by immersion. Turbot of both sizes (distributed in Tanks G1A, G2A, G1B and G2B) were exposed to virus at a concentration of 10^5 $TCID_{50}$ ml^{-1} in a total volume of 3 l for 3 h. As negative controls, four tanks (one per size and temperature) containing ten fish each were also set up using MEM with no virus.

Intraperitoneal infection. Individuals of 5 g (distributed in tanks $G1B_{ip}$ and $G2B_{ip}$) were injected i.p. with an inoculum volume of 100 µl representing a dose of 10^5 $TCID_{50}$ per fish. Two control groups of 10 fish (one at each temperature) were injected with 100 µl of MEM with no virus.

The fish were monitored daily for signs of disease and mortality. Every five days eight fish of each size and temperature were sampled over a month (6 sample points). Fish were euthanized with a MS-222 overdose and eyes and brain were aseptically removed from each individual. Tissues of five individuals were pooled and used for virological analysis (cell culture and Real-time PCR), whereas the other three were subjected to histopathological examination. Fish surviving at the end of the experiment were also

191 examined for the presence of virus. Control fish were also tested to detect IPNV, IHNV,
192 VHSV and NNV at the end of the trial.

193

194 ***2.4.****Betanodavirus detection by SYBR Green real time PCR.*

195 Synthesis of cDNA was performed as previously described. Real time-PCR reactions
196 were carried out in a final volume of 50 µl, containing 200 nM of each primer SnodR1
197 F/R (5'-TCCAAAAGAAAGAAGCATAC-3'/5'-TCCAAAAGAAAGAAGCATAC-3')
198 and 2 µl of cDNA in iQ™ SYBR® Green Supermix (Bio-Rad). Following an initial 15
199 min denaturation/activation step at 95˚C, the mixture was subjected to 45 cycles of
200 amplification (denaturation for 15 s at 95˚C, annealing and extension for 15 s at 60˚C)
201 in a CFX96™ Real-time PCR detection system (BioRad). Generation of PCR products
202 was monitored after each extension step at 59ºC by measuring the fluorescence of
203 double-stranded DNA binding SYBR green dye.

204 ***2.5.*** *Histopathological examination.*

205 Brain samples were fixed in neutral phosphate buffered 10% formalin. Standard
206 processing procedures for light microscopy (LM) were followed. After embedding in
207 paraffin, 6 µm sections were cut, mounted on glass slides and stained with haematoxylin
208 and eosin (H&E).

209 **3. Results**

210 Internal examination of affected turbot showed abundant ascitic fluid and an anaemic
211 liver with petechial haemorrhages.

212 ***3.1.****Virology.*

213 There was no evidence of CPE in first passage on any of the cell lines used 15 days
214 after inoculation. After a blind passage E-11 monolayers showed partial disintegration
215 and rounded, refractile granular cells with vacuoles could be observed. The viral isolate
216 was confirmed as betanodavirus by RT-PCR and named SpPm-IAusc1586.10
217 As it is shown in Fig 1 fish were only positive for betanodavirus by RT-PCR (specific
218 amplification product of 420 bp). This result was confirmed by nested PCR (specific
219 amplification product of 156 bp). In the nested-PCR analysis fish were positive also for
220 IPNV (Fig 1)

221 ***3.2.*** *Nucleotide sequence comparison and phylogenetic analysis.*

222 Complete ORF sequences of both genomic segments from the betanodavirus turbot
223 isolate SpPm-IAusc1586.10 were analysed and compared with those available in the
224 international data base belonging to the four genotypes of betanodavirus (SJNNV,
225 RGNNV, BFNNV and TPNNV, see Table 1). The coding sequence for protein A
226 (RNA1) (acc no KC696563) showed an identity of 98.55±1.06 with RGNNV genotype,
227 and a similar value (99.2±0.55) in the RNA 2 (KC696562) coding region, whereas the
228 similarity with the remaining genotypes was clearly lower (82.2±0.0 to 83.1±0.28 for
229 RNA1 and 79.2 ±0.14 to 81.75±0.35 for RNA2). Results of phylogenetic analysis
230 performed on RNA1 and RNA2 indicated that the turbot isolate clustered within the
231 RGNNV genotype (Fig 2).

232 ***3.3.****Experimental infection.*

233 In the first trial performed with the betanodavirus isolate fish did not display any signs
234 of disease and no mortality was recorded in the 30 days of the duration of the
235 experiment. No virus was recovered in cell culture from the fish sacrificed at the end of
236 the experiment and virus was detected by real time with high C_T values (>30) at both
237 temperatures.

In the second trial fish were sacrificed every 5 days over a month to be subjected to virological (cell culture and Real-time PCR) and histopathological analysis. As in the first trial fish were completely asymptomatic. No natural mortalities were observed regardless of the route of infection (immersion or i.p. injection) or the temperature assayed (15 and 18ºC). However, in this set of experiments it was possible to recover the virus in cell culture and the level of detection was higher than in the previous experiment. The results obtained from the sacrificed fish as well as from those surviving at the end of the experiments are shown in Table 2. Virus was detected by Real time and recovered in cell culture from the fish infected by i.p. at almost any sampling time at both 15 and 18ºC. Regarding the fish infected by immersion, the best results were obtained at 18ºC and in 2 g turbot, in which NNV was detected by Real time at 5, 10, 15 and 20 days p.i and viral isolation was obtained in the first three sampling points. At 15ºC betanodavirus were detected and recovered in cell culture from 2 g fish only at 15 days p.i. The results obtained from 5 g fish showed that virus was detected mainly in the middle-final sampling points (15-30 days) at both temperatures, but viral recovery was only attained at 18ºC.

The histopathological analysis of the brain revealed that none of the infected individuals showed vacuolization or any other pathological change.

Neither mortalities nor signs of disease were observed in the non-challenged fish, and no virus was detected in these control fish at the end of the trials.

4. Discussion

In the present study we report the first isolation of a betanodavirus strain, belonging to the RGNNV genotype, from juvenile turbot showing unspecific symptoms and very low mortality. The presence of an IPNV-type virus was also revealed in the fish tissues, but

263 but only after nested-PCR, suggesting a very low viral load. In addition, *Vibrio* spp was
264 also isolated from some of the individuals.
265 Experimental infections were conducted in order to determine the pathogenicity of the
266 betanodavirus strain for juvenile turbot. Challenges were performed by immersion at
267 two different temperatures: 15ºC (water temperature of turbot water tanks when viral
268 isolation was performed) and 18ºC (the highest water temperature reached in summer in
269 our area, which is more appropriate for betanodavirus growth). First challenges were
270 terminated after a month, with no mortalities recorded and no symptoms of disease
271 observed. In addition no virus was recovered in cell culture, and viral detección by real
272 time RT-PCR was effective at quite high C_T values, suggesting a relatively low viral
273 load. New challenges were performed in order to study viral replication in the fish
274 tissues at shorter time intervals; two different sizes of turbot were used and sacrificed
275 every 5 days, over a period of 30 days. An intraperitoneal infection was also carried out
276 with the biggest fish. As in the first experiments, fish did not show signs of VNN and no
277 mortalities were observed. These results indicated that a NNV isolate belonging to
278 RGNNV genotype does not induce clinical disease in this fish species. However, SpPm-
279 IAusc15688.11 isolate was recovered from some of the fish infected at both
280 temperatures. Furthermore, viral recovery from experimental infections showed that the
281 virus may persist in surviving fish for up to 25 days following waterborne exposure and
282 30 days after i.p. inoculation. These results together with the fact that the virus was
283 originally isolated from turbot juveniles demonstrated that turbot can be infected with
284 this genotype, despite the apparent lack of clinical disease. Similar findings have been
285 previously observed in other fish species infected with different betanodavirus isolates.
286 In this sense, as mentioned in a review by Munday et al. (2002), in a taxonomic study
287 by Nishizawa et al. (1997) two nodaviruses were used (a SJNNV-type isolate from red

sea bream *Pagrus major*, and a BFNNV-type from Pacific cod *Gadus macrocephalus*) that had not been reported to cause disease in their host species. Other authors reported a betanodavirus isolation from healthy gilthead sea bream *Sparus aurata* (Castric et al., 2001), and performed experimental infections which did not cause any clinical signs.

The lack of clinical disease observed in the juvenile turbot analysed in this study contrasts with previous demonstration of susceptibility to TNV genotype in a natural outbreak (Johansen et al., 2004), and to SJNNV and BFNNV genotypes in experimental infections (Husgard et al., 2001; Sommerset et al., 2003; Montes et al., 2010). These results suggest that the genotype can be determinant in the likelihood of inducing clinical disease in turbot by an NNV strain. Similar genotype-specific trends have been reported for different VHSV genotypes and different fish species (Skall et al., 2004; Snow et al., 2005).

It could be argued that the temperature used in the challenges was not the most appropriate for NNV and the reason behind the lack of clinical symptoms. It has been reported that rearing water temperatures influences the development of VER and that high temperatures (24-28ºC) are probable predisposing factors of the disease (Arimoto et al., 1994; Fukuda et al., 1996; Tanaka et al., 1998; Breuil et al., 2001). However, Tanaka et al. (1998) reported that redspotted grouper *Epinephelus septemfasciatus* was susceptible to the virus at 16ºC and, although mortality was lower than that observed at higher temperatures, infected fish showed abnormal swimming. These results demonstrate that a high temperature is not a determinant factor for the development of clinical symptoms. In our experimental infections with the smallest fish (2g), the raising of the temperature led to an increase in the number of individuals positive for NNV, suggesting than the fish were more susceptible to viral replication. However, this result

312 was not observed in older fish, which seems to indicate that fish size is an important
313 factor in viral replication, like in the fish affected by VER (Munday et al., 2002).
314
315 **5. Conclusion**
316 The isolation of a NNV- RGNNV type strain from turbot with no signs of VER and
317 the results of the experimental challenges performed demonstrate that turbot can be
318 infected with this genotype although it does not induce clinical disease. Turbot
319 susceptibility/resistance to VER may be genotype specific, since other genotypes have
320 been reported to cause natural or experimental mortalities in this species. The evidence
321 in this study seems to indicate that the existence of a RGNNV genotype reservoir in the
322 wild fish in the area represents a relatively low risk to the turbot farming industry.
323
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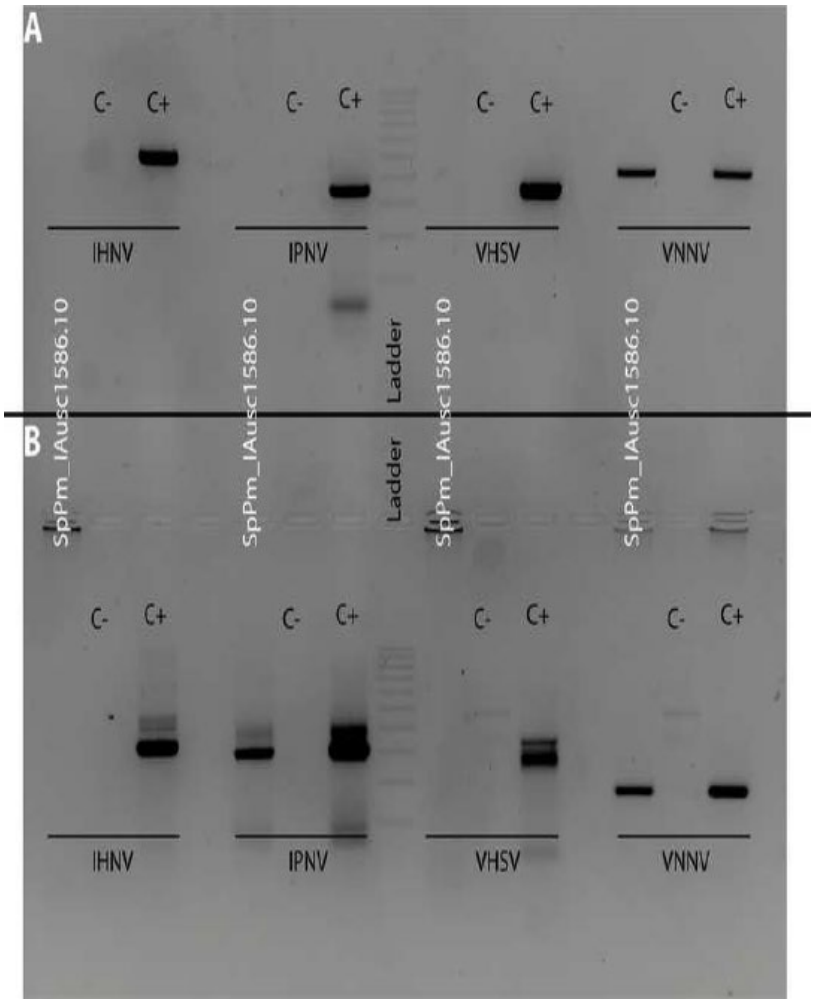
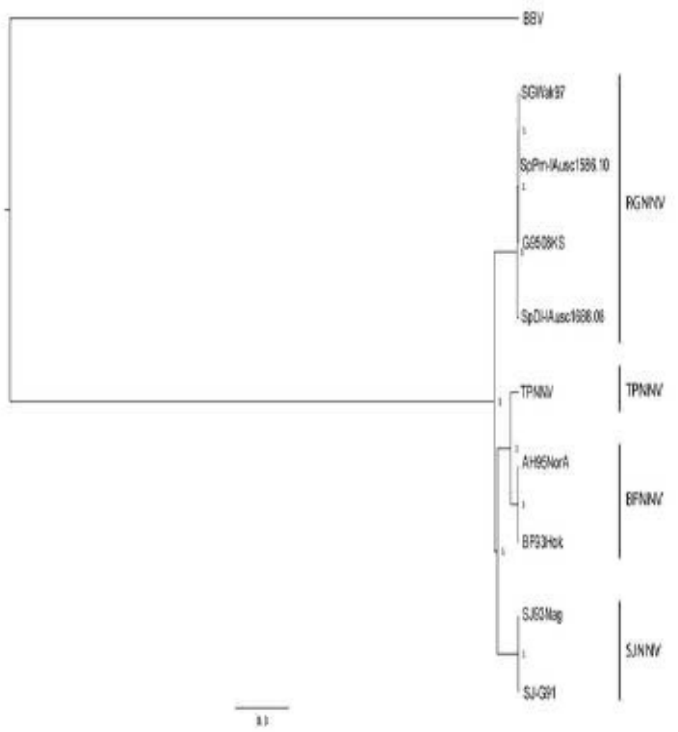
425 Figures



426

427 Figure 1. Results of the RT-PCR and Nested-PCR analysis performed to the juvenile
428 turbot. A) RT-PCR amplification products (10 µl) analyzed on an agarose gel stained by
429 ethidium bromide, B) Nested PCR performed using 3 µl from the RT-PCR reactions.
430 DNA ladder (L), Simply load 100bp DNA ladder (Lonza): 10 fragment ranging from
431 100 bp to 1,000 bp in 100 bp increments

2A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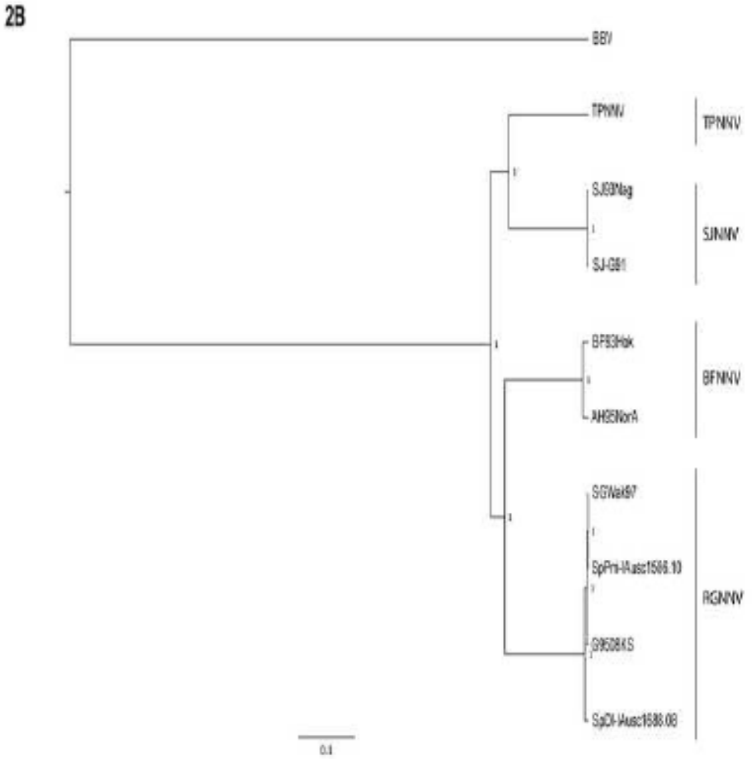
BFNNV
SJ93Mag
SJ-G91
SJNNV

432

2B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TPNNV
SJ93Mag
SJ-G91
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BF83Hok
AH95NorA
BFNNV
SGWak97
SpPrn-IAusc1586.10
GB508KS
SpDI-IAusc1688.08
RGNNV

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Figure 2 Phylogenetic relationships among the turbot betanodavirus isolate and reference strains from the four genotypes. (a) RNA1-based phylogeny and (b) RNA2-based phylogeny, both corresponding to the ORF sequences. Phylogeny was inferred using Bayesian inference of phylogeny using BEAST 1.7.5 (Bayesian MCMC analysis of molecular sequences) and employing the GTR+G (RNA1) and HYK+G (RNA2) models. Numbers indicate Bayesian PPs obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the first 50 000 generations. Sequence of alphanodavirus BBV was used as an outgroup. Major betanodavirus groups are labelled according to Nishizawa et al. (1997). Bars, 0.3 nucleotide substitutions per site