

1 Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic  
2 outbreaks in farmed fish species provides evidence for genetic reassortment.

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25 Abstract

26 Betanodaviruses, belonging to the family *Nodaviridae*, are the etiological agents of a  
27 devastating neuropathological condition that affects marine fish worldwide. The genome of  
28 betanodaviruses consists of two single-stranded, positive-sense RNA molecules. The larger  
29 genomic segment, RNA1, encodes the RNA-dependent RNA polymerase (RdRP), also named  
30 protein A. The smaller segment, RNA2 encodes the capsid protein (CP). Betanodavirus are  
31 classified in four genotypes on the basis of the comparison of a variable sequence of RNA2:  
32 striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), red-  
33 spotted grouper nervous necrosis virus (RGNNV), and barfin flounder nervous necrosis virus  
34 (BFNNV) types. Sequencing of the full coding region of both genomic segments of seven  
35 betanodavirus strains isolated from different farmed species in Spain and Portugal revealed that  
36 six of them were reassortants, exhibiting a RGNNV RNA1 and a SJNNV RNA2. Analysis of  
37 sequences of reassortant strains at both genomic and protein level revealed the existence of  
38 differences with type strains of both genotypes. These differences were higher in the polymerase  
39 sequence, which is remarkable because viral structural proteins generally diverge more rapidly  
40 than non-structural proteins. Changes in two amino acids observed in the SJNNV capsid protein  
41 might be involved in the colonization of new host species by these reassortant strains. In  
42 addition, a more extensive phylogenetic analysis, including partial sequences of both RNA  
43 segments of other 16 Iberian nodavirus, confirmed the existence of reassortment between  
44 RGNNV and SJNNV.

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46

47 Introduction

48 The family *Nodaviridae* contains small (25-30 nm), non-enveloped, icosahedral RNA viruses,  
49 grouped in two genera, *Alphanodavirus* and *Betanodavirus*, which infect a wide range of insects  
50 and fish, respectively (Schneemann *et al.*, 2005). Betanodaviruses are the etiological agents of  
51 the disease known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy  
52 (VER), a devastating neuropathological condition that affects marine fish worldwide (Munday  
53 *et al.*, 2002).

54 The genome of betanodaviruses consists of two single-stranded, positive-sense RNA molecules.  
55 The larger genomic segment, RNA1 (3.1 Kb), encodes the RNA-dependent RNA polymerase  
56 (RdRP) of approximately 100 kDa, also named protein A (Nagai & Nishizawa 1999; Tan *et al.*,  
57 2001). The smaller segment, RNA2 (1.4 Kb), encodes the capsid protein (CP) of about 42 KDa  
58 (Delsert *et al.*, 1997; Nagai & Nishzawa 1999). In addition, a subgenomic RNA3 is synthetized  
59 during RNA replication from the 3' terminus of RNA1. The RNA3 encodes a protein called B2  
60 (Sommerset & Nerland, 2004; Iwamoto *et al.*, 2005) which suppresses cellular RNA  
61 interference (Iwamoto *et al.*, 2005; Fenner *et al.*, 2006 a, b). To date a limited number of full-  
62 length betanodavirus RNA1 sequences have been published (Nagai & Nishizawa, 1999; Tan *et*  
63 *al.*, 2001; Sommerset & Nerland, 2004; Okinaka & Nakai, 2008), whereas complete nucleotide  
64 sequences of RNA 2 are available for a higher number of isolates.

65 RNA2 segment contains a highly variable region (the so called T4 region) (Nishizawa *et al.*,  
66 1995), which encodes the C-terminal half of the capsid protein. Comparison of nucleotide  
67 sequence of the T4 region was used by Nishizawa *et al* (1997) to classify betanodaviruses in  
68 four genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus  
69 (TPNNV), red-spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous  
70 necrosis virus (BFNNV). This classification has currently been used to genotype new isolates in  
71 different countries (Aspehaug *et al.*, 1999; Starkey *et al.*, 2000; Dalla Valle *et al.*, 2001; Skliris  
72 *et al.*, 2001; Johnson *et al.*, 2002; Chi *et al.*, 2003; Gagné *et al*, 2004; Johansen *et al.*, 2004). A  
73 different nomenclature for the genotypes was proposed by Thiéry *et al* (2004), the groups

74 named I, II, III and IV corresponded to RGNNV, BFNNV, TPNNV and SJNNV types,  
75 respectively. The Iberian betanodaviruses (IBNNV) genotyped so far by our group have been  
76 included within SJNNV (IV) clade (Cutrín *et al.*, 2007).

77 Recently, Toffolo *et al.* (2007) have reported the first phylogenetic study of betanodavirus  
78 isolates using partial sequences of both RNA1 and RNA2. Nucleotide sequence analyses of both  
79 segments maintained the distinction of the four recognized genotypes, although topologies of  
80 the phylogenetic trees differed in the position of the BFNNV clade. In addition, those authors  
81 reported the detection of two reassortant strains, which exhibited a RNA1 typed as SJNNV and  
82 a RNA2 belonging to RGNNV type. Other authors used a longer sequence of both segments to  
83 study the phylogenetic relationships among betanodavirus infecting cod in Norway, and did not  
84 observe discrepancies in the clades obtained neither reassortants (Nylund *et al.*, 2008).

85 In the present study the full coding regions of RNA1 and RNA2 of seven betanodavirus strains  
86 isolated from diseased farmed fish in the Iberian Peninsula were sequenced and subjected to  
87 phylogenetic analysis. In addition, a more extensive phylogenetic study, including partial  
88 sequences of both RNA segments of other 16 Iberian nodavirus, was performed. Furthermore,  
89 deduced amino acid sequences corresponding to the protein A (RdRP) of the seven isolates was  
90 subjected to an evolutionary trace analysis, and the secondary structure was predicted.

91

## 92 **METHODS**

93 **Virus strains and viral propagation.** Betanodavirus strains analyzed in this work are listed in  
94 table 1. This collection included seven isolates obtained from different farmed fish species (sea  
95 bass, *Dicentrarchus labrax*, gilthead sea bream, *Sparus aurata* and Senegalese sole, *Solea*  
96 *senegalensis*) showing disease symptoms, and one reference strain, SJ93Nag (kindly provided  
97 by Dr T. Nishizawa, Japan), which were propagated in cell culture. In addition, sixteen  
98 nodavirus sequences obtained after RT-PCR amplification of RNA extracted from infected  
99 tissues were used in some phylogenetic analysis. Nucleotide sequences of betanodaviruses  
100 available in the international databases were used for comparative purposes.

101 Viral isolates were propagated in SSN-1 cell line (Frerichs *et al.*, 1996) grown in L-15 medium  
102 containing 5% foetal bovine serum. Inoculated cells were incubated at 20°C up to a maximum  
103 of 10 days. Culture fluids were recovered when cytopathic effect (CPE) became extensive, and  
104 clarified by centrifugation at 3000×g for 15 min at 4°C. Aliquots of the viral suspension were  
105 stored frozen (-80°C) until use.

106 **RNA extraction.** Total RNA was extracted directly from viral suspensions, using RNeasy®  
107 Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were  
108 resuspended in nuclease-free water (Promega), quantified by spectrophotometry at 260 nm  
109 using a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies) and stored at -20° C.

110 **DNA synthesis.** The reaction was performed in a MyCycler™ Thermal Cycler (Bio-Rad) by  
111 mixing 2.5 ng µl<sup>-1</sup> of random primers (Promega) and 10 ng µl<sup>-1</sup> of viral RNA in nuclease-free  
112 water, heating at 95°C for 5 min and incubating at 4°C for at least 1 min. Then a reverse  
113 transcription mixture containing 10 U µl<sup>-1</sup> of Superscript III RT (Invitrogen), 2 U µl<sup>-1</sup> of  
114 RNaseOUT Recombinant RNase inhibitor (Invitrogen), 0.5 mM of each dNTP and 0.05 M of  
115 DTT in 1× First Strand buffer was added and incubated at 25° C for 10 min followed by 50 min  
116 at 50 ° C. Prior to PCR amplification, the reaction was inactivated by heating at 85° C for 5 min.

117 **PCR amplification and cDNA sequencing.** The sequence of the coding regions of both RNA  
118 segments from the seven IBNNV isolates (SpSa-IAusc156.03, SpSs-IAusc160.03, PtSs-  
119 IAusc573.04, PtSa-IAusc61.05, PtSa-IAusc74.05, SpDI-IAusc1688.08 and SpSs-IAusc1974.08)  
120 was determined using the primer walking approach. A panel of 9 sets of primers (6 for RNA1  
121 and 3 for RNA2) were designed to amplify fragments of approximately 400- to 650-bp,  
122 corresponding to overlapping regions of the betanodavirus genome (Table 2). For the remaining  
123 16 strains, sequencing was performed on fragments of 531 and 403 nt of RNA1 and RNA2,  
124 respectively (Table 2). PCR amplification was accomplished by transferring 2 µl of cDNA to a  
125 PCR mix with 1.25 U of GoTaq® DNA polymerase (Promega) and 0.5 µM of the specific  
126 primer set. Following an initial 4 min denaturation step at 94° C, the mixture was subjected to  
127 40 cycles of amplification (denaturation for 30 s at 94° C, annealing for 30 s at 55° C, and

128 extension for 30 s at 68° C). A final extension period of 10 min at 68° C preceded storage of the  
129 reaction product at 4° C. PCR products were subjected to electrophoresis through a 1.5%  
130 SeaKem® LE agarose gel (FMC Bioproducts) and the specific bands were purified using a  
131 Montage DNA Gel Extraction Device filtrate vial (Millipore). Automated sequencing was  
132 performed using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter). The sequences  
133 were confirmed at least twice with each one of the sense and antisense primers. The genomic  
134 coding regions of SJNag93, a strain previously characterized by Iwamoto *et al.* (2001), were re-  
135 sequenced in order to check the accuracy of the sequencing process.

136 **Sequence analysis.** The sequences were edited using DNASTAR Lasergene® v.7.1 SeqMan II  
137 and EditSeq (DNASTAR) and subjected to multiple sequence alignment using the DNASTAR  
138 Lasergene v.6 MegAlign program. Reference strains belonging to the four genotypes of  
139 betanodavirus were also included in the alignment. The divergence among sequences was  
140 determined using the DnaSP software (Rozas *et al.*, 2003).

141 **Phylogenetic algorithms and methods.** A first phylogenetic analysis was performed on the  
142 complete ORFs of both segments, using as reference complete sequences available in the Gene  
143 Bank (Table 2). However, owing to the absence of complete genome sequences of  
144 betanodavirus strains belonging to TPNNV genotype, a second phylogenetic analysis was  
145 performed on shorter fragments of RNA1 (933 nt, between positions 198 to 1130 referred to  
146 SJNag93, acc. no. AB056571) and RNA2 (836 nt, from position 186 to 1021, referred to  
147 SJNag93, acc. no. AB056572). Trees were constructed by Bayesian inference of phylogeny using  
148 Mr. Bayes 3.1 (Ronquist & Huelsenbeck, 2003) as implemented in TOPALi v2.5 (Milne *et al.*  
149 2009). This software chose K80 + G as optimal model of evolution for both datasets. Two  
150 Markov chains were run for 1,000,000 generations and Bayesian posterior probabilities (PP)  
151 were obtained from the 50% majority rule consensus of trees sampled every 100 generations  
152 after removing the 50,000 first generations. Sequences of two alphanodaviruses – BBV [black  
153 beetle virus, accession no. NC\_001411 (RNA1) and NC\_002037 (RNA2)] and NOV [nodamura  
154 virus, NC\_002690 (RNA1) and NC\_002691 (RNA2)]– were used as an outgroup to produce  
155 rooted trees.

156 In order to include a higher number of Iberian betanodavirus sequences (16), a shorter region of  
157 both RNA segments was used for additional phylogenetic studies. These sequences  
158 corresponded to a 531 positions long fragment of RNA1, located within the amplification  
159 product of primers F7/R7 (Table 2) and 403 nt of RNA 2, corresponding almost completely to  
160 the amplification product of primers F2/R3. For simplicity, these trees were inferred according  
161 to the Neighbour Joining algorithm as implemented in TOPALi v2. The statistical support for  
162 each node of both trees was assessed using bootstrapping based on 1000 replicates.

163 **Recombination analysis.** Intragenic recombination was investigated using the RDP3 v 3.34  
164 software (Martin *et al.*, 2005), which allows a multiple approach. We performed recombination  
165 analyses by applying Chimaera, GENECONV, Maximum  $\chi^2$ , Bootscan and RDP algorithms.

166 **Evolutionary trace.** Aligned protein sequences were submitted to the evolutionary trace server  
167 of the Baylor College of Medicine for construction of a phylogenetic tree based on a distance  
168 matrix from the PHYLIP package (v3.6beta) computing the consensus sequences. The  
169 sequences on different branches of the tree were grouped into different evolutionary classes  
170 according to their degree of similarity (Lichtarge *et al.*, 1996, Carlsson *et al*, 2009). To generate  
171 these classes, an evolutionary time cutoff line (ETC) divided the phylogenetic tree into 10  
172 partitions, PI to PX, in order of increasing divergence. A class was formed by sequences  
173 originating from the same node on the phylogenetic tree and shared the evolutionary time cutoff  
174 line that created the partition. This ensured that the most similar sequences belonged to the same  
175 class whereas the most distant ones belonged to different classes. In a given partition, sequences  
176 within different classes were separately aligned, and the resultant aligned classes were  
177 compared to obtain their consensus residues, called the trace residues, for that partition. Three  
178 types of trace residues were identified: those that remained invariant across all classes were  
179 designated ‘absolutely conserved residues’, those that remained strictly conserved within a class  
180 but differed between classes were designated ‘class-specific residues’, whereas trace residues  
181 that were not conserved within any class were designated ‘neutral’.

182 **Secondary structure prediction.** The amino acid sequence of protein A from reference  
183 RGNNV strain and IBNNV isolates were submitted to the Protein structure prediction server at  
184 the University College London (Bryson *et al.*, 2005) to be analyzed by PSIPRED method (Jones  
185 *et al.*, 1999) and to the Network protein sequence analysis (NPS@) server at the University of  
186 Lyon (Combet *et al.* 2000).

## 187 **RESULTS**

### 188 **Nucleotide sequence comparison**

189 The sequence of SJ93Nag, strain used to check the accuracy of the sequencing process, was  
190 identical to the original one. The nucleotide sequences of the seven isolates under study were  
191 analyzed and compared with those available in the international data base belonging to the four  
192 genotypes of betanodavirus (SJNNV [3 strains], RGNNV [3 strains], BFNNV [3 strains] and  
193 TPNNV [1 strain]; see Table 1). Comparison with TPNNV was made on the longest sequences  
194 available in the data bases, which corresponded to fragments of 933 nt and 836 nt for RNA1 and  
195 RNA2, respectively. The coding sequence for protein A (RNA1) of the seven IBNNV isolates  
196 showed an identity of  $97.1 \pm 0.5$  with RGNNV genotype, whereas the similarity with the  
197 remaining genotypes was lower (80.4 to 82.1%) (Table 3). As expected the lowest similarity  
198 was observed with alphanodavirus with a percentage of  $43.1 \pm 0.8$ . The comparison of the  
199 sequences corresponding to the coat protein (RNA 2) showed differences among the isolates.  
200 Thus, one of them (SpDI-IAusc1688.08) had a high similarity with RGNNV type (98.6%),  
201 whereas the other six isolates showed the highest similarity with SJNNV genotype ( $98.3 \pm 0.1$ ).  
202 As observed with the polymerase, the similarity of the coat protein of these Iberian  
203 betanodavirus with alphanodavirus was very low ( $35.9 \pm 0.3$ ). All the sequences have been  
204 deposited in the Gene Bank with accession numbers FJ 803911 to 803923 and FJ829452 (see  
205 Table 1).

206 Pairwise comparisons with RGNNV type strain (SGWak93) identified a total of 140 different nt  
207 in the coding sequence of RNA1 (2949 nt), which represents 4.7% of the sequence. The number  
208 of differences ranged from 65 nt, observed with the isolate SpSs-IAusc1688.08, to 103 nt found

209 with isolate SpSs-IAusc1974.08, which showed the lowest identity percentage (96.5%) with the  
210 reference strain (data not shown); 32 of these substitutions were shared by the seven strains, and  
211 81 by six of them (excluding SpDI-1688.08), which represents 2.7% of the coding region.  
212 Pairwise comparison of RNA2 was performed with reference strains of genotypes SJNNV and  
213 RGNNV. Differences of six of the isolates with SJNNV type strain ranged from 16 to 19 nt (8  
214 changes shared by the six strains; 0.8% of the coding region) (data not shown), whereas isolate  
215 DI-IAusc1688.08 showed a mismatch of 12 positions with RGNNV type strain.

### 216 **Phylogenetic analysis**

217 Due to the lack of complete sequences of TPNNV in the international database and in order to  
218 include representative strains of the four genotypes a first phylogenetic analysis was performed  
219 on RNA1 and RNA2 fragments of 933 and 836 nt, respectively, which were the longest  
220 available sequences for a TPNNV strain (Fig 1). An additional phylogenetic analysis performed  
221 on the complete ORFs was carried out excluding TPNNV, providing similar results (data not  
222 shown)

223 Phylogenetic analysis based on RNA1 is presented in figure 1A. The tree, constructed using two  
224 alphanodaviruses (BBV and NOV) as outgroup, showed the four established genotypes with PP  
225 values of 1. A sister group relationship between TPNNV + BFNNV (clades III and II) and  
226 SJNNV (clade IV) genotypes was observed, whereas the RGNNV (group I) remained separate.  
227 The seven Iberian isolates clustered with RGNNV strains, although two subgroups were  
228 distinguished with the maximum PP: subgroup Ia included 6 out of 7 Iberian nodavirus, whereas  
229 subgroup Ib included the reference strains and one of the Iberian nodavirus, strain SpDI-  
230 IAusc1688.08

231 The tree performed using RNA2 sequences (Fig 1B) also confirmed the four clades but depicted  
232 different relationships. The tree topology indicated that clades I and II can be considered sister  
233 groups. In addition, it revealed the existence of a similar relationship between clades IV and III,  
234 as well. One of the isolates analyzed in this study, DI-IAusc16808 (obtained from sea bass)  
235 clustered with RGNNV reference strains. The other 6 isolates, obtained from Senegalese sole  
236 and gilthead sea bream, were included within SJNNV genotype (clade IV), although different

237 subtypes could be recognized: Subtype IVa contained one Spanish isolate (SpSs-IAusc1974.08)  
238 whereas the Japanese reference strains and the remaining Iberian isolates were included in  
239 subtype IVb (both groups clustering separately).

240 Restricting the analysis to a shorter fragment of both segments allowed inclusion of 16  
241 additional IBNNV sequences obtained from gilthead sea bream and Senegalese sole (accession  
242 numbers GQ131547 to GQ131571). The results obtained were identical to those described  
243 above: all RNA1 sequences clustered with RGNNV genotype, and all RNA2 sequences were  
244 included within SJNNV genotype, excepting SpDI-1688.08, that clustered with RGNNV (Fig  
245 2).

#### 246 **Evolutionary trace (ET) analysis**

247 Alignment of deduced amino acid sequences of the capsid protein from the 6 isolates showing a  
248 SJNNV-type RNA2 revealed the existence of 5-6 differences with the reference strains of this  
249 genotype (data not shown). Three of these differences were shared by the 6 isolates (positions  
250 20, 247 and 270), which represents 0.9% of the amino acid composition of the capsid protein.  
251 However, comparison of the amino acid sequences of the protein A (typed as RGNNV) with the  
252 reference strain SGWak97 showed from 20 to 22 aminoacid substitutions, 19 of them shared by  
253 all the isolates (1.9% of the polymerase amino acid residues). Analysis of the isolate SpDI-  
254 IAusc1688.08, typed as RGNNV using both segments, revealed only three and four differences  
255 in capsid and protein A composition, respectively, when compared with the RGNNV type strain  
256 (data not shown).

257 To determine if the different residues observed in protein A characterized the evolutionary  
258 process of the Iberian nodavirus, the ET algorithm was used. A phylogenetic tree was divided  
259 into 10 partitions (I to X), sorting the sequences into different groups or classes, where each  
260 class consist of a cluster of similar sequences originating from a given node within that  
261 partition. Two branches were observed in the first partition (Fig. 3), branch 1 contained  
262 reference strain SGWak97 and strain SpDI-IAusc1688.08, whereas branch 2 consisted of the  
263 remaining IBNNV sequences. Branch 2 was further subdivided in the following partitions in

264 order of increasing divergence. Thus, partition IX distinguished SpSs-IAusc1974.08 from the  
265 other strains and in partition X two subgroups were observed.

266 The ET analysis of the six IBNNV strains showing a RGNNV-type RNA1 and a SJNNV-type  
267 RNA-2 revealed that 14 amino acid substitutions observed in the protein A, from a total of 20-  
268 22, were class-specific (Fig. 4).

### 269 **Predicted secondary structures of protein A**

270 In order to examine if the 14 class-specific mutations could affect the protein A structure,  
271 secondary structure predictions were performed using two methods. Both methods agreed in the  
272 prediction and revealed only one change in the secondary structure (Fig 5), affecting the “ $\alpha$ - $\beta$ -  
273  $\alpha$ ” arrangement between positions 258 and 299, by including an additional  $\beta$ -strand and  
274 reducing the length of the final  $\alpha$ -helix. This change could be caused by some of the nearby  
275 point mutations: position 218 (Phe→Leu, both non-polar, hydrophobic), 223 (Met→Leu, both  
276 non-polar, hydrophobic) 238 (Phe →Tyr, non-polar, hydrophobic → polar, hydrophilic) and 289  
277 (Ala →Thr non-polar, hydrophobic → polar, hydrophilic).

### 278 **Recombination analysis**

279 The multiple approach used to detect possible recombination events did not provide positive  
280 results. Although some possible recombination events were located by Max chi and Chimaera  
281 methods involving strain SpDI-IAusc1688.08, none of these events were found to be significant.

### 282 **Discussion**

283 Betanodavirus have been classified into four types, designated SJNNV, TPNNV, RGNNV and  
284 BFNNV using a partial sequence of the RNA2, the T4 region (Nishizawa *et al.*, 1997). Those  
285 types exhibit a different capability to infect fish species. In this sense, RGNNV shows the  
286 broadest host range and causes disease in a variety of warm-water fish species; BFNNV is  
287 restricted to cold-water marine fish species, and TPNNV infects only one or two species  
288 (Munday *et al.*, 2002). Regarding to SJNNV type, although for several years it was considered  
289 to be restricted to a few species present in Japanese waters (Nishizawa *et al.*, 1997, Munday *et*  
290 *al.* 2002) in the last years it has been found in Senegalese sole (Thiéry *et al.* 2004, Cutrín *et al.*

291 2007) as well as gilthead sea bream and sea bass cultured in the Iberian peninsula (Cutrín *et al.*  
292 2007).

293 Phylogenetic analysis based on the full coding region of both genomic segments of the 7 Iberian  
294 nodavirus under study was intended to be performed including strains of the four established  
295 genotypes. However, no representative strains of TPNNV genotype could be included, since,  
296 although it has been reported the sequencing of the complete genome of the type strain of this  
297 genotype (acc. no. EU236148 and EU236149) (Okinaka & Nakai, 2008), these sequences are  
298 not available in the data bank. Therefore, in order to include representative strains of the four  
299 genotypes, the phylogenetic analysis was performed on a shorter region of both segments. The  
300 phylograms obtained using both RNAs maintained the clusters corresponding to the different  
301 genotypes. However, six of the seven IBNNV strains exhibited markedly different genetic  
302 position depending on which segment was analyzed. Same results were obtained when  
303 phylograms were performed on complete coding regions sequences in absence of the TPNNV  
304 strain.

305 RNA2-derived phylogenies indicated that six strains clustered with SJNNV reference trains,  
306 whereas only one, SpDI-IAusc1688.08, was grouped with RGNNV genotype. These results  
307 indicated that most of the strains belonged to the SJNNV type as reported previously by our  
308 group in a more extensive study which included typing (based on the T4 region) of 31 Iberian  
309 betanodavirus (Cutrín *et al.* 2007). However, analysis of RNA1 depicted completely different  
310 phylogenetic relationships, and the seven isolates clustered with RGNNV genotype. These  
311 results indicate a natural reassortment between strains of SJNNV and RGNNV genotypes in six  
312 out of the seven strains analyzed. In addition, the analysis of a shorter and different region of  
313 both segments indicated that other additional 16 strains, 7 of them previously classified as  
314 SJNNV type from a RNA2 analysis (Cutrín *et al.* 2007), are actually RGNNV/SJNNV  
315 reassortants.

316 Chimeric betanodaviruses using SJNNV and SGNNV (belonging to RGNNV type) strains have  
317 been constructed previously in laboratory, thus demonstrating the feasibility of reassortment  
318 between both genotypes (Iwamoto *et al.*, 2004). However, the evidence of natural genetic

319 reassortment occurring between different strains of betanodavirus in a fish has been reported  
320 only once (Toffolo *et al.*, 2007). In that study 2 strains from a group of 27 betanodavirus  
321 isolates harboured a RNA1 segment of SJNNV type and a RNA2 belonging to RGNNV type.  
322 Both reassortant strains were obtained from symptomatic sea bass. These data indicate that both  
323 combinations between genomic segments of SJNNV and RGNNV genotypes are successful,  
324 and allowed resultant reassortant strains to produce disease. Interestingly, from the results of  
325 that study and the present one, a certain relationship between the type of reassortant and the  
326 susceptible host species seems to exist: SJNNV/RGNNV affecting to sea bass and  
327 RGNNV/SJNNV to Senegalese sole and gilthead sea bream.

328 Genome segment reassortment is a complex phenomenon and there must be some requirement  
329 for the exchange to be effect. Analysis of the reassortant strains of the present study indicate  
330 differences in both genomic segments when compared with the reference strains of each  
331 genotype that could be involved in the success of the exchange process. Substitutions observed  
332 in the six strains represented 0.7% and 2.7% of the coding region of RNA1 and RNA2,  
333 respectively. The analysis of the nucleotide mutations in the capsid protein ORF of the  
334 reassortant strains revealed that 6 of the 8 changes (75%) observed in all strains render these SJ  
335 segments more similar to the RG segment that they could have replaced. The same analysis  
336 performed on the RdRp ORF indicated that 34 of the 81 changes (42%) render these RG  
337 segments more similar to the RNA1 of SJNNV type strain. If the acceptance of a heterologous  
338 segment depended on the number of mutations that render it more similar to the homologous  
339 one, we could speculate that our reassortant strains came from a RGNNV strain which accepted  
340 a RNA2 from a SJNNV strain. However, for some reovirus reassortants it has been described  
341 that only a few mutations are necessary for the acceptance of a heterologous segment (Joklik &  
342 Roner, 1995). More recent results indicate that the incorporation of an engineered ssRNA into  
343 the genome of reoviruses depends critically on three consecutive nucleotides of the 5'UTR  
344 (Roner *et al.* 2004). Unfortunately we have not sequenced the complete UTRs of both genomic  
345 segments from all reassortant strains. Therefore, although we have observed some nucleotide  
346 changes in these regions (data not shown) it is not possible to reach any reliable conclusion.

347 The total number of nucleotide mutations observed in genome segments of the reassortant  
348 strains resulted in 5-6 amino acid substitutions in the capsid protein (3 shared by the 6 isolates)  
349 and 20-22 in the polymerase (19 shared by all the isolates), which represents 0.9% and 1.9 % of  
350 the respective sequences. This is remarkable since structural proteins generally diverge more  
351 rapidly than non-structural ones, presumably in response to a greater environmental pressure.  
352 Despite the changes in the amino acid composition of the reassortant RdRp (most of them  
353 described as class specific by the evolutionary trace analysis), most of the protein sequence  
354 revealed characteristic conservation patterns. In fact, all the polymerase motifs identified in  
355 betanodavirus (the acidic motif [aa 585-590], the SG.T motif [aa 646-651], the GDD motif [aa  
356 686-688], the basic motif [aa 712] and an aromatic residue preceded by a basic sequence [aa  
357 808]) (Nagai & Nishizawa, 1999; Tan *et al.*, 2001) were considered absolutely conserved  
358 regions by this analysis. In addition, as expected the predicted secondary structure of protein A  
359 did not reveal changes in the polymerase domain of the reassortant strains and the differences  
360 were restricted to the N-terminal third.

361 It has been reported that the capability of fish nodavirus to infect different fish species is  
362 controlled by the coat protein (Iwamoto *et al.*, 2004). In addition, it is known that even a small  
363 number of amino acid substitutions in the capsid proteins can have dramatic effects on the host  
364 specificity of different animal viruses (Baranowski *et al.*, 2001). All the reassortant strains  
365 exhibited a slightly modified SJNNV capsid, with 3 different amino acid positions in all strains  
366 (the differences raised to a maximum of 6 in some strains). One of this changes observed in  
367 residue 270 was encoded by the nucleotide triplet 737-739, included in the sequence 695-765,  
368 described by Ito *et al.* (2008) as a host specific determinant. Other change in the amino acid  
369 sequence (residue 270) was also observed in the C-terminal side of the capsid protein. These  
370 results confirm that C-terminal protruding domains of capsid protein are involved in host  
371 specificity as reported previously (Iwamoto *et al.*, 2004, Ito *et al.*, 2008). However, the other  
372 change was observed in the N-terminal third of the protein. Tang *et al.* (2002) suggested that the  
373 N-terminal region from residues 1 to 82 of malabaricus grouper necrosis virus (belonging to  
374 RGNNV genotype) may contribute to the neutralization of the negative charge of the viral

375 nucleic acid in virus assembly and RNA packaging. The significance of the mutations in this  
376 area in the reassortant strains needs to be clarified. The reassortant isolates obtained from  
377 Senegalese sole also shared the changes at residues 247 and 279, but showed additional  
378 differences at positions 293 or 298, depending on the strain. These results suggest that a few  
379 changes in the C-terminal domain of the capsid protein might favour the colonization of new  
380 hosts. Regarding this shift to new hosts it is important to remember that all IBNNV sequences  
381 analyzed in this study obtained from sea bream and Senegalese sole, including some previously  
382 classified as SJNNV, are reassortant strains.

383 The present study provides interesting insights into the possible evolution and colonization of  
384 new hosts by betanodavirus strains. The aminoacid substitutions in the capsid protein possibly  
385 have allowed broadening the range of susceptible host of SJNNV type. Further studies will be  
386 necessary to clarify the significance of the changes observed in the polymerase of the  
387 reassortant strains.

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517

Table 2.-Primers used for sequencing.

Primer	Nucleotide Sequence (5'-3')	Position*		Size (bp)
		RNA 1	RNA 2	
<i>Used for sequencing of the 7 IBBNV isolates</i>				
NNVs1_1f	TAACATCACCTTCTTGCTCT	1-20		613
NNVs1_1r	AACCATCCTTAACTTCTCC	595-613		
NNVs1_2f	TTTGGGTGTTCCGATATTGTTGTA	546-569		568
NNVs1_2r	CAGTTCGTATGCAGTGC GGATGTT	1090-1113		
NNVs1_3f	ATTCGAGAACATCCGCACTG	1082-1102		615
NNVs1_3r	ACTTGACGTTTTGGGTGTGC	1677-1696		
NNVs1_4f	ACATCTCAACCGTTCGCACACC	1661-1683		552
NNVs1_4r	TCAAGTCAAAGCCAAGGGAAGAAG	2189-2212		
NNVs1_5f	GCTCCTCACCAACTGCT	2172-2190		653
NNVs1_5r	CATCATCACCTGGAAGAGC	2806-2824		
NNVs1_6f	TCGCATTAGACGGGGAGAT	2699-2717		408
NNVs1_6r	GCCGAAGCGTAGGACAGC	3089-3106		
NNVs2_1f	AAGTCAAAATGGTACGCAAAGGTG		20-43	527
NNVs2_1r	CTCCCGGTTGAGGTCCAGAGA		525-546	
NNVs2_2f	GCTTCCTGCCTGATCCAAC TGA		404-425	568
NNVs2_2r	GGCGCCCCAACTGTGAATGTCT		950-971	
NNVs2_3f	CGGTTACTGATTTTCG TTC		732-752	655
NNVs2_3r	ACATGGCGGTGGTGCTC		1370-1386	
<i>Used for partial sequencing of 16 additional IBNNV RNAs</i>				
F7	ATATCACGATGAGTTC ACTA	1584-1603 <sup>†</sup>		642 <sup>‡</sup>
R7	CGATTCACTATTTTCAAGTC	2206-2225 <sup>†</sup>		
F2	CGTGTCAGTCATGTGTCGCT		615-634 <sup>§</sup>	427 <sup>□</sup>
R3	CGAGTCAACACGGGTGAAGA		1022-1041 <sup>§</sup>	

519 \* Positions of the primers based on the published sequence of SJ93Nag strain (accession no.

520 AB056571 and AB056572)

521 <sup>†</sup>According to Nishizwa *et al.*, 1994

522 <sup>‡</sup>For sequencing only a length of 531 nt was employed

523 <sup>§</sup> According to Nevarez *et al.*, 2004

524 <sup>□</sup> For sequencing only a length of 403 nt was employed

525

526

527 Table 3. Sequence similarities between the full coding sequence of both genomic segments of 7  
528 Iberian isolates, reference strains of the four genotypes of betanodavirus and two  
529 alphanodavirus

Genotype	RNA-1		RNA-2 <sup>†</sup>
	7 IBNNV isolates*	6 IBNNV isolates	SpDI-IAusc1688.08
SJNNV <sup>‡</sup>	82.1 ± 0.2 <sup>§</sup>	98.3 ± 0.1	79.5 ± 0.1
RGNNV	97.1 ± 0.5	79.2 ± 0.3	98.6 ± 0.2
BFNNV	80.4 ± 0.1	77.2 ± 0.2	82.5 ± 0.3
TPNNV <sup>□</sup>	82.1 ± 0.13	80.3 ± 0.2	78.8
ALPHANODAVIRUS <sup>¶</sup>	43.1 ± 0.8	35.9 ± .2	35.8 ± 0.8

530 \*SpSa-IAusc156.03, SpSs-IAusc160.03, PtSs-IAusc573.04, PtSa-IAusc61.05, PtSa-IAusc74.05,  
531 and SpSs-IAusc1974.08 and SpDI-IAusc 1688.08.

532 <sup>†</sup>For RNA2 comparison, the results from the isolate yielding the highest differences are shown  
533 in a separate column

534 <sup>‡</sup>Reference strains from each genotype: SJNag93, SJNNV and SJ-G91 from SJNNV,  
535 SGWak97, GGNV and G9508KS from RGNNV, AH99NorA, Ac06NorT and BF93Hok from  
536 BFNNV and TPKag93 from TPNNV.

537 <sup>§</sup>Data are expressed as average nucleic acid identity percentage ± standard deviation

538 <sup>□</sup>Comparison was performed on a 933 nt for RNA1 and 836 nt for RNA2 because ORF  
539 sequences were unavailable at GenBank.

540 <sup>¶</sup>BBV (black beetle virus) and NOV (nodamura virus)

541

542

543 **Figure legends**

544 Figure 1. Phylogenetic relationships among 7 Iberian betanodavirus isolates and reference  
545 strains from the four genotypes. A. RNA1 based phylogeny (933 nt, between positions 198 to  
546 1130 referred to SJNag93, acc. no. AB056571). B. RNA2 based phylogeny (836 nt, from  
547 position 186 to 1021, referred to SJNag93, acc. no. AB056572)

548 Phylogeny was inferred using Mr. Bayes 3.1 as implemented in TOPALi v2. Numbers indicate  
549 bayesian posterior probabilities (PP) obtained from the 50% majority rule consensus of trees  
550 sampled every 100 generations after removing the 50,000 first generations. Sequences of two  
551 alphanodaviruses BBV (black beetle virus) and NOV (nodamura virus) were used as an  
552 outgroup. Mayor betanodavirus groups are labelled according to Nishizawa *et al.* (1997)\* and  
553 Thiéry *et al.* (2004)<sup>†</sup>.

554 Figure 2. Phylogenetic analysis performed on 16 Iberian betanodavirus sequences. A. RNA1  
555 based phylogeny (531nt). B. RNA2 based phylogeny (403 nt)

556 Phylogeny was inferred by the Neighbor Joining method included in PAUP version 4.0 beta.  
557 Two alphanodavirus, BBV (black beetle virus) and NOV (nodamura virus) were used as  
558 outgroup. Bootstrap values are presented as percentages of 1,000 resamplings. The length of  
559 horizontal branches is proportional to nucleotide substitutions. Mayor betanodavirus groups are  
560 labelled according to Nishizawa *et al.* (1997) \* and Thiéry *et al.* (2004)<sup>†</sup>.

561 Figure 3. Evolutionary tree based on a distance matrix from the PHYLIP package generated  
562 from the Baylor college of medicine server of the aminoacid sequences of protein A from the  
563 six reassortant strains and reference strain SGWak97. The partition lines I to X indicate the  
564 extent of divergence of the sequences

565 Figure 4. Evolutionary traces for partitions I to X from the evolutionary tree shown in Fig 3.  
566 The trace residues belonging to a given partition occur in the horizontal row corresponding to  
567 the partition. ‘Absolute conserved residues’ are surrounded by boxes, ‘class-specific residues’  
568 are denoted by an X, and ‘neutral residues’ are marked with a dash (-) sign.

569 Figure 5. Secondary structure predictions for the protein A sequences of the IBNNV isolates  
570 (six reassortants and one RGNNV) and SGWak97 reference strain as provided by NPS@  
571 (Combet *et al.*, 2000). Predicted  $\alpha$ - helices of four or more residues are shown in blue,  $\beta$ -strands  
572 in red and random coils or turns as horizontal purple line.

Figure 1A

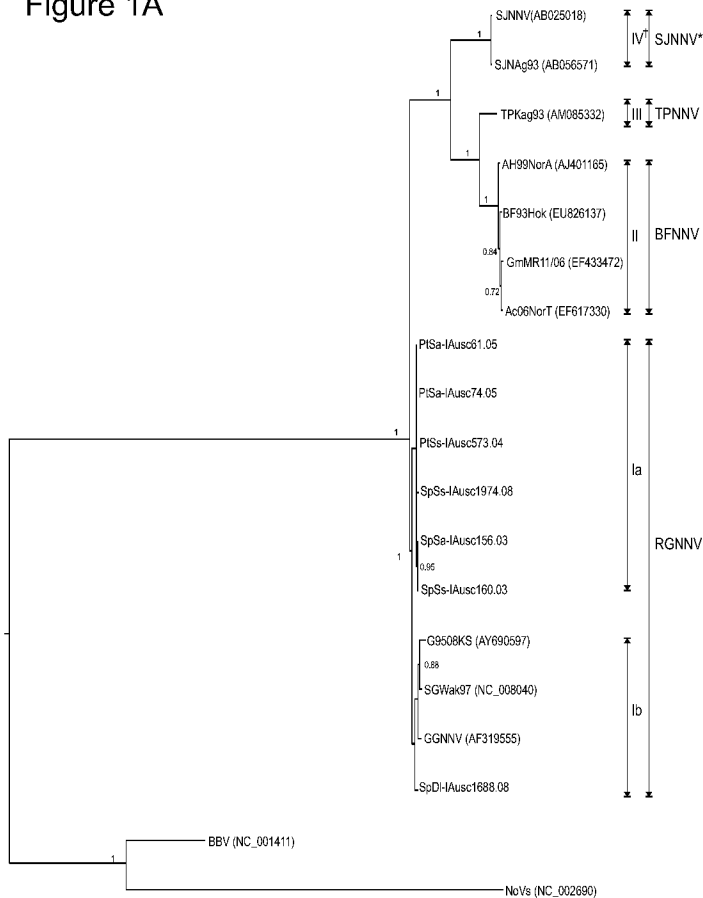


Figure 1B

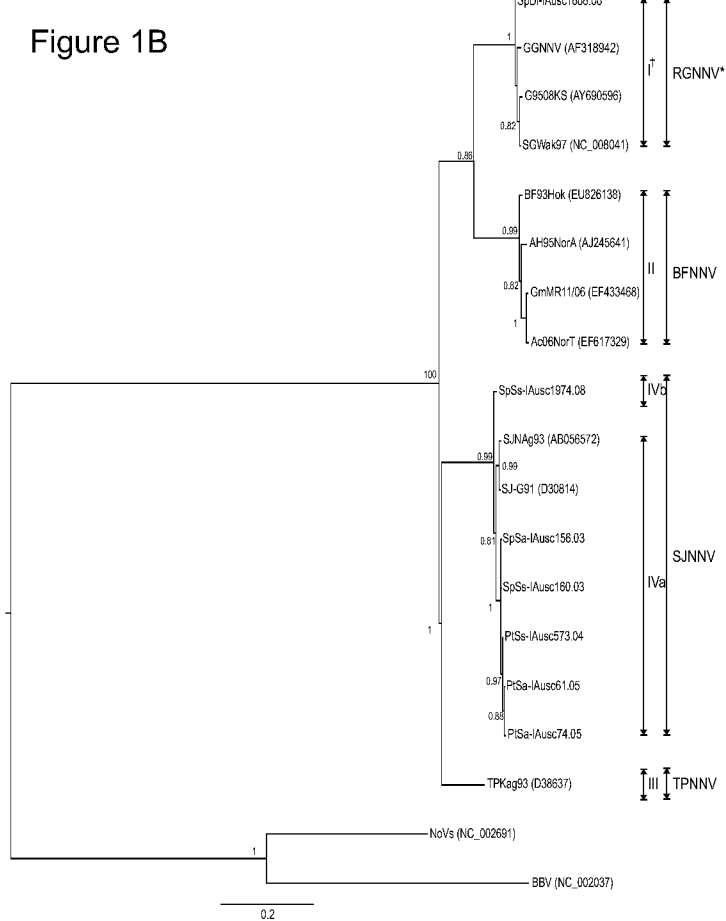


Figure 2A

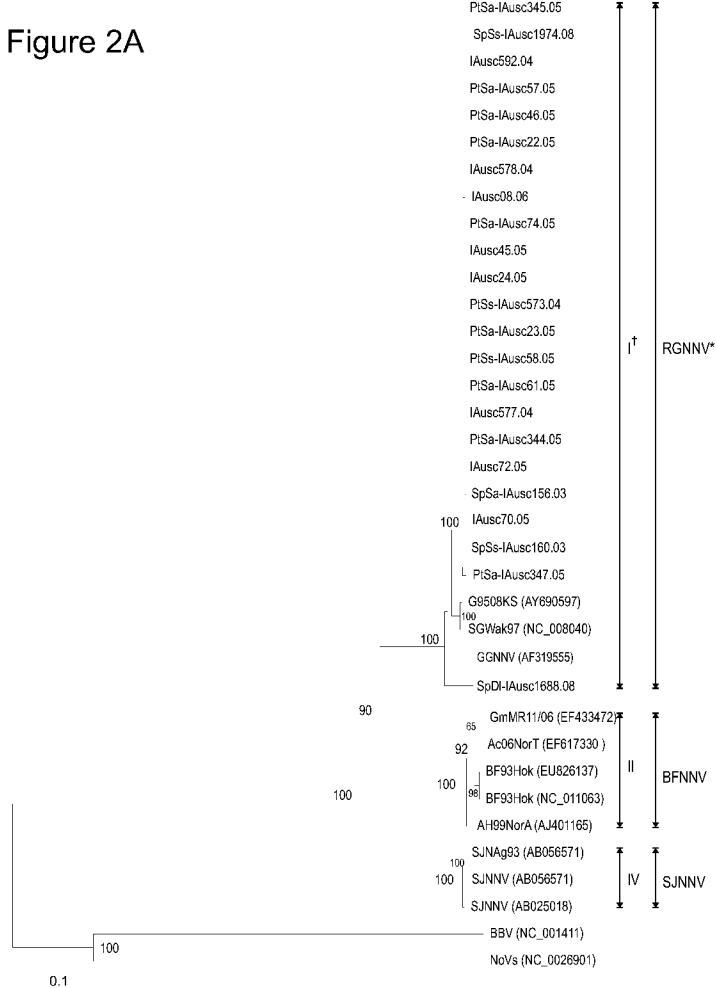


Figure 2B

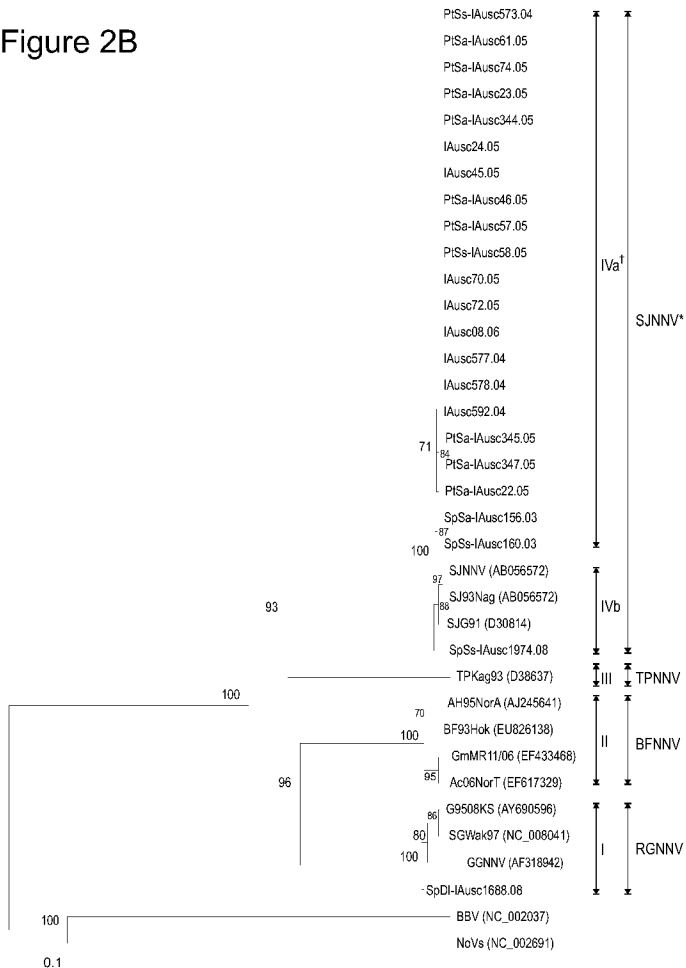






Figure 5

