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

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Sequencing of the full coding region of both genomic segments of seven betanodavirus strains isolated from different farmed species in Spain and Portugal revealed that six were reassortants, exhibiting a red-spotted grouper nervous necrosis virus (RGNNV)-type RNA1 and a striped jack nervous necrosis virus (SJNNV)-type RNA2. Analysis of sequences of reassortant strains at both the genomic and protein levels revealed the existence of differences compared with type strains of both genotypes. These differences were greater in the polymerase sequence, which is remarkable because viral structural proteins generally diverge more rapidly than non-structural proteins. Changes in two amino acids observed in the SJNNV capsid protein might be involved in the colonization of new host species by these reassortant strains. In addition, a more extensive phylogenetic analysis, including partial sequences of both RNA segments of 16 other Iberian nodaviruses, confirmed the existence of reassortment between RGNNV and SJNNV.

INTRODUCTION

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

The genome of betanodaviruses consists of two single-stranded, positive-sense RNA molecules. The larger genomic segment, RNA1 (3.1 kb), encodes the RNA-dependent RNA polymerase (RdRp) of approximately 100 kDa, also named protein A (Nagai & Nishizawa, 1999; Tan et al., 2001). The smaller segment, RNA2 (1.4 kb), encodes the capsid protein of about 42 kDa (Delsert et al., 1997; Nagai & Nishizawa, 1999). In addition, a subgenomic RNA3 is synthesized during RNA replication from the 3’ terminus of RNA1. RNA3 encodes a protein called B2 (Sommerset & Nerland, 2004; Iwamoto et al., 2005), which suppresses cellular RNA interference (Iwamoto et al., 2005; Fenner et al., 2006a, b). To date, a limited number of full-length betanodavirus RNA1 sequences have been published (Nagai & Nishizawa, 1999; Iwamoto et al., 2001, 2004; Tan et al., 2001; Sommerset & Nerland, 2004; Okinaka & Nakai, 2008), whereas complete nucleotide sequences of RNA2 are available for a larger number of isolates.

RNA2 segment contains a highly variable region (the so-called T4 region) (Nishizawa et al., 1995), which encodes the C-terminal half of the capsid protein. Comparison of nucleotide sequences of the T4 region was used by Nishizawa et al. (1997) to classify betanodaviruses into 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). This classification is currently used to genotype new isolates in different countries (Aspehaug et al., 1999; Starkey et al., 2000; Dalla Valle et al., 2001; Skliris et al., 2001; Johnson et al., 2002; Chi et al., 2003; Gagné et al., 2004; Johansen et al., 2004). A different nomenclature for the genotypes was proposed by Thiéry et al. (2004), with the groups named I, II, III and IV corresponding to RGNNV, BFNNV, TPNNV...
The betanodavirus strains and viral propagation.

METHODS

Virus strains and viral propagation. The betanodavirus strains analysed in this work are listed in Table 1. This collection included seven isolates obtained from different farmed fish species (sea bass, Dicentrarchus labrax; gilthead sea bream, Sparus aurata; Senegalese sole, Solea senegalensis) showing abnormal swimming behaviour and moderate to high mortalities. All isolates were obtained from larvae except for SpDi-IAusc1688.08 (from juveniles of sea bass of approximately 15 g) and SpSs-IAusc1974.08 (from juveniles of Senegalese sole of approximately 20 g). The samples (whole individuals when using larvae and a pool of eyes and brain when larger fish were used) were processed and inoculated in duplicate onto SSN-1 and SAF-1 cells grown on 24-well-plates as described previously (Oliveira et al., 2008). Infected monolayers were incubated at 20 and 25 °C, and examined daily for the presence of a cytopathic effect (CPE). Every 10 days, one of the wells inoculated with each sample was subjected to a second or to blind passages (up to two passages), whereas the other well was maintained for up to 30 days. In addition, 16 nodavirus sequences obtained after RT-PCR amplification of RNA extracted from infected tissues were used in some phylogenetic analyses. One reference strain, SJ93Nag (kindly provided by Dr T. Nishizawa, Hiroshima University, Japan) was propagated in cell culture and used to check the accuracy of the sequencing process. Nucleotide sequences of betanodaviruses available in international databases were used for comparative purposes.

Viral isolates were propagated in SSN-1 cells (Frerichs et al., 1996) grown in 1-1.5 medium containing 5 % fetal bovine serum. Inoculated cells were incubated at 20 °C for up to 10 days. Culture fluids were recovered when the CPE became extensive, and clarified by centrifugation at 3000 g for 15 min at 4 °C. Aliquots of the viral suspension were stored frozen (−80 °C) until use.

RNA extraction. Total RNA was extracted directly from viral suspensions using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA samples were resuspended in nuclease-free water (Promega), quantified by spectrophotometry at 260 nm using a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies) and stored at −20 °C.

DNA synthesis. The reaction was performed in a MyCycler thermal cycler (Bio-Rad) by mixing random primers (2.5 ng µl−1; Promega) and viral RNA (10 ng µl−1) in nuclease-free water, heating at 95 °C for 5 min and incubating at 4 °C for at least 1 min. A reverse transcription mixture containing Superscript III reverse transcriptase (10 U µl−1; Invitrogen), RNaseOUT Recombinant RNase inhibitor (2 U µl−1; Invitrogen), 0.5 mM each dNTP and 0.05 M dithiothreitol in 1× First Strand buffer (Invitrogen) was added and incubated at 25 °C for 10 min, followed by 50 min at 50 °C. Prior to PCR amplification, the reaction was inactivated by heating at 85 °C for 5 min.

PCR amplification and cDNA sequencing. The sequence of the coding regions of both RNA segments from the seven IBNNV isolates (SpSa-IAusc156.03, SpSs-IAusc160.03, PtSa-IAusc573.04, PtSa-IAusc61.05, PtSa-IAusc74.05, SpDI-IAusc1688.08 and SpSs-IAusc1974.08) was determined using a primer-walking approach. A panel of nine sets of primers (six for RNA1 and three for RNA2) were designed to amplify fragments of approximately 400–650 bp, corresponding to overlapping regions of the betanodavirus genome (see Supplementary Table S1, available in JGV Online). For the remaining 16 strains, sequencing was performed on fragments of 531 and 403 nt of RNA1 and RNA2, respectively (Table 2). PCR amplification was accomplished by transferring 2 µl cDNA to a PCR mix containing 1.25 U GoTaq DNA polymerase (Promega) and 0.5 µM of the specific primer set. Following an initial 4 min denaturation step at 94 °C, the mixture was subjected to 40 cycles of amplification (30 s at 94 °C, 30 s at 55 °C and 30 s at 68 °C). A final extension period of 10 min at 68 °C preceded storage of the reaction product at 4 °C. PCR products were subjected to electrophoresis through a 1.5 % SeaKem LE agarose gel (FMC Bioproducts) and the specific bands were purified using a Montage DNA Gel Extraction Device filtrate vial (Millipore). Automated sequencing was performed using a CEQ 8000 Genetic Analysis System (Beckman Coulter). The sequences were confirmed at least twice with each of the sense and antisense primers. The genomic coding regions of SJNag93, a strain previously characterized by Iwamoto et al. (2001), were resequenced to check the accuracy of the sequencing process.

Sequence analysis. The sequences were edited using DNASTAR Lasergene v7.1 SeqMan II and EditSeq and subjected to multiple sequence alignment using the DNASTAR Lasergene v6 MEGALIGN program. Reference strains belonging to the four genotypes of betanodavirus were also included in the alignment. The divergence among sequences was determined using DnaSP software (Rozas et al., 2003).

Phylogenetic algorithms and methods. A first phylogenetic analysis was performed on the complete open reading frames (ORFs) of both segments, using complete sequences available in GenBank as reference (Table 2). However, owing to the absence of complete genome sequences of betanodavirus strains belonging to the TPNNV genotype, a second phylogenetic analysis was performed on shorter fragments of RNA1 (933 nt, positions 198–1130 with reference to SJNag93, GenBank accession no. AB056571) and RNA2 (836 nt, positions 186–1021, with reference to SJNag93, GenBank...
accession no. AB056572). Trees were constructed by Bayesian inference of phylogeny using Mr Bayes v3.1 (Ronquist & Huelsenbeck, 2003) as implemented in TOPALi v2.5 (Milne et al. 2009). This software chose K80 + G as the optimal model of evolution for both datasets. Two Markov chains were run for 1 000 000 generations and Bayesian posterior probabilities (PPs) were obtained from the 50 % majority rule consensus of trees sampled every 100 generations after removing the first 50 000 generations. Sequences of two alphanodaviruses – black beetle virus [BBV; GenBank accession nos NC_001411 (RNA1) and NC_002037 (RNA2)] and nodamura

Table 1. Betanodavirus isolates and sequences used in this study

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Year</th>
<th>Source</th>
<th>Country</th>
<th>Reference</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpSa-IAus156.03</td>
<td>2003</td>
<td>Gilthead sea bream</td>
<td>Spain</td>
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<td>FI803916 FI803921</td>
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<td>Senegalese sole</td>
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<td>This report</td>
<td>FI803914 FI803920</td>
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<td>FI803912 FI803918</td>
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<td>FI803913 FI803919</td>
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<td>SpDl-IAus1688.08</td>
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<td>Sea bass</td>
<td>Spain</td>
<td>This report</td>
<td>FI803915 FI8039452</td>
</tr>
<tr>
<td>SpSa-IAus1974.08</td>
<td>2008</td>
<td>Senegalese sole</td>
<td>Spain</td>
<td>This report</td>
<td>FI803917 FI803922</td>
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<td>SJ93Nag (SJNNV)*</td>
<td>1993</td>
<td>Striped jack</td>
<td>Japan</td>
<td>Iwamoto et al. (2001)</td>
<td>AB056571† AB056572†</td>
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**RT-PCR products used for sequence analysis**

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<td>Portugal</td>
<td>This report‡; Cutrin et al. (2007)§</td>
</tr>
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<td>2004</td>
<td>Senegalese sole</td>
<td>Portugal</td>
<td>This report; Cutrin et al. (2007)</td>
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<td>592.04</td>
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<td>This report</td>
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<td>This report; Cutrin et al. (2007)</td>
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<td>PtSa-IAus08.06</td>
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<td>Gilthead sea bream</td>
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**Reference strains used for sequence analysis**

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<th>Virus isolate</th>
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<th>Source</th>
<th>Country</th>
<th>Reference</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGWak97 (RGNNV)</td>
<td>1997</td>
<td>Red-spotted grouper</td>
<td>Japan</td>
<td>Iwamoto et al. (2004)</td>
<td>NC_008040† NC_008041†</td>
</tr>
<tr>
<td>G9508KS (RGNNV)</td>
<td>2002</td>
<td>Red-spotted grouper</td>
<td>Japan</td>
<td>Lee et al. (2002)</td>
<td>AY690566† AY690567†</td>
</tr>
<tr>
<td>GNNV (RGNNV)</td>
<td>2001</td>
<td>Greasy grouper</td>
<td>Singapore</td>
<td>Tan et al. (2001)</td>
<td>AF319555† AF318942†</td>
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<tr>
<td>AH99NorA (BFNNV)</td>
<td>1995</td>
<td>Atlantic halitut</td>
<td>Norway</td>
<td>Sommerset &amp; Nerland (2004); Grotmol et al. (2000)</td>
<td>AJ40116† AJ245641†</td>
</tr>
<tr>
<td>Ac06NorT (BFNNV)</td>
<td>2007</td>
<td>Atlantic cod</td>
<td>Norway</td>
<td>Patel and others, unpublished data¶</td>
<td>EF617330† EF617329†</td>
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<td>BF93Hok (BFNNV)</td>
<td>1993</td>
<td>Barfin flounder</td>
<td>Japan</td>
<td>Nishizawa et al. (1995)</td>
<td>EU826137† EU826138†</td>
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<td>GmMR11/06 (BFNNV)</td>
<td>2008</td>
<td>Atlantic cod</td>
<td>Norway</td>
<td>Nylund et al. (2008)</td>
<td>EF433472† EF433468†</td>
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<tr>
<td>TPKag93 (TPNNV)</td>
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<td>Tiger puffer</td>
<td>Japan</td>
<td>Toffolo et al. (2007); Nishizawa et al. (1995)</td>
<td>AM085332§ D38637§</td>
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*Betanodavirus genotype.
†Complete sequence.
‡Partial sequence obtained in the present study.
§Partial sequence.
**Complete ORF.
¶Direct submission to GenBank.
Table 2. Sequence similarities between the full coding sequence of both genomic segments of seven Iberian isolates, reference strains of the four genotypes of betanodavirus and two alphanodavirus

Data are expressed as percentage mean ± SD nucleic acid identity.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RNA-1</th>
<th>RNA-2*</th>
<th>SpDI-IAusc1688.08</th>
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<tr>
<td>SJNNV‡</td>
<td>82.1 ± 0.2</td>
<td>98.3 ± 0.1</td>
<td>79.5 ± 0.1</td>
</tr>
<tr>
<td>RGNNV</td>
<td>97.1 ± 0.5</td>
<td>79.2 ± 0.3</td>
<td>98.6 ± 0.2</td>
</tr>
<tr>
<td>BFNNV</td>
<td>80.4 ± 0.1</td>
<td>77.2 ± 0.2</td>
<td>82.5 ± 0.3</td>
</tr>
<tr>
<td>TPNNV‡</td>
<td>82.1 ± 0.13</td>
<td>80.3 ± 0.2</td>
<td>78.8</td>
</tr>
<tr>
<td>Alphanodavirus‖</td>
<td>43.1 ± 0.8</td>
<td>35.9 ± 0.2</td>
<td>35.8 ± 0.8</td>
</tr>
</tbody>
</table>

*For RNA2 comparison, the results from the isolate yielding the highest differences are shown in a separate column.
‡SpSa-IAusc156.03, SpSs-IAusc160.03, PtTs-IAusc573.04, PtSa-IAusc61.05, PtSa-IAusc74.05, SpSs-IAusc1974.08 and SpDI-IAusc1688.08.
§Reference strains from each genotype: SJ93Nag, SJNNV and SJ-G91 from the SJNNV genotype; SGWal97, GGNV and G9508KS from the RGNV genotype, AH99NorA, Ac06NorT, GmMR11/06 and BF93Hok from the BFNNV genotype and TP93Kag93 from the TPNNV genotype.
§Comparison was performed on a 933 nt for RNA1 and 836 nt for RNA2 because ORF sequences were unavailable in GenBank.
‖BBV and NoV.

In order to include a larger number of Iberian betanodavirus sequences (16), a shorter region of both RNA segments was used for additional phylogenetic studies. These sequences corresponded to a 531 nt fragment of RNA1, located within the amplification product of primers F7/R7 (Table 2) and 403 nt of RNA2, corresponding almost completely to the amplification product of primers F2/R3. For simplicity, these trees were inferred according to the neighbour-joining algorithm as implemented in TOPALi v2. The statistical support for each node of both trees was assessed using bootstrapping based on 1000 replicates.

**Recombination analysis.** Intragenic recombination was investigated using the RdRp v3.34 software (Martin et al., 2005), which allows a multiple approach. We performed recombination analyses by applying CHIMAERA, GENECOV, MAXIMUM χ², BOOTSCAN and RDP algorithms.

**ET analysis.** Aligned protein sequences were submitted to the evolutionary trace server of the Baylor College of Medicine for construction of a phylogenetic tree based on a distance matrix from the PHYLIP package (v3.6f) computing the consensus sequences. The sequences on different branches of the tree were grouped into different evolutionary classes according to their degree of similarity (Lichtarge et al., 1996; Carlsson et al., 2009). To generate these classes, an evolutionary time cut-off line divided the phylogenetic tree into ten partitions, PI–PX, in order of increasing divergence. A class was formed by sequences originating from the same node on the phylogenetic tree and shared the evolutionary time cut-off line that created the partition. This ensured that the most similar sequences belonged to the same class, whereas the most distant ones belonged to different classes. In a given partition, sequences within different classes were aligned separately, and the resultant aligned classes were compared to obtain their consensus sequences, called the trace residues, for that partition. Three types of trace residue were identified: those that remained invariant across all classes were designated ‘absolutely conserved residues’ and those that remained strictly conserved within a class but differed between classes were designated ‘class-specific residues’, whereas trace residues that were not conserved within any class were designated ‘neutral’.

**Secondary-structure prediction.** The amino acid sequences of RdRp from reference strain RGNV and IBNNV isolates were submitted to the protein structure prediction server at University College London, UK (Bryson et al., 2005), to be analysed by PSIPRED (Jones, 1999) and to the network protein sequence analysis (NPS@) server at the University of Lyon, France (Combet et al., 2000).

**RESULTS**

**Nucleotide sequence comparison**

The sequence of SJ93Nag, the strain used to check the accuracy of the sequencing process, was identical to the original sequence. The nucleotide sequences of the seven isolates under study were analysed and compared with those available in GenBank belonging to the four genotypes of betanodavirus (SJNNV, three strains; RGNV, three strains; BFNNV, four strains; TPNNV, one strain; see Table 1). Comparison with TPNNV was made on the longest sequences available in the database, which corresponded to fragments of 933 and 836 nt for RNA1 and RNA2, respectively. The coding sequence for RdRp (RNA1) of the seven IBNNV isolates showed a sequence identity of 97.1 ± 0.5 % with the RGNV genotype, whereas the similarity with the remaining genotypes was lower (80.4–82.1 %) (Table 2). As expected, the lowest similarity was observed with alphanodaviruses (43.1 ± 0.8 %). Comparison of the sequences corresponding to the coat protein (RNA2) showed differences among the isolates. Thus, one of them (SpDI-IAusc1688.08) had a high similarity with the RGNV type (98.6 %), whereas the other six isolates showed the highest similarity with the SJNNV genotype (98.3 ± 0.1 %). As observed with the RdRp protein, the similarity of the coat protein of these Iberian betanodaviruses with alphanodaviruses was very low (35.9 ± 0.3 %).
Pairwise comparisons with the RGNNV type strain (SGWak97) identified a total of 140 different nucleotides in the coding sequence of RNA1 (2949 nt), which represented 4.7% of the sequence. The number of differences ranged from 65 nt observed with isolate SpSs-IAusc1688.08 to 103 nt found with isolate SpSs-IAusc1974.08, which showed the lowest identity percentage (96.5%) with the reference strain (data not shown); 32 of these substitutions were shared by the seven strains and 81 by six of them (excluding SpDl-1688.08), which represented 2.7% of the coding region. Pairwise comparison of RNA2 was performed with reference strains of the genotypes SJNNV and RGNNV. Differences for six of the isolates with the SJNNV type strain ranged from 16 to 19 nt (eight changes shared by the six strains; 0.8% of the coding region; data not shown), whereas isolate SpDl-IAusc1688.08 showed a mismatch of 12 positions with the RGNNV type strain.

Phylogenetic analysis

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

Phylogenetic analysis based on RNA1 is presented in Fig. 1(a). The tree, constructed using two alphanodaviruses (BBV and NoV) as the outgroup, showed the four established genotypes with PP values of 1. A sister-group relationship between the TPNNV + BFNNV (clades III and II) and SJNNV (clade IV) genotypes was observed, whereas the RGNNV (group I) genotype remained separate. The seven Iberian isolates clustered with the RGNNV strains, although two subgroups were distinguished with the maximum PP: subgroup Ia included six of the seven Iberian nodaviruses, whereas subgroup Ib included the reference strains and one of the Iberian nodaviruses, strain SpDl-IAusc1688.08.

The tree performed using RNA2 sequences (Fig. 1b) also confirmed the four clades, but depicted different relationships. The tree topology indicated that clades I and II could be considered sister groups. In addition, it revealed the existence of a similar relationship between clades III and IV. One of the isolates analysed in this study, SpDl-IAusc1688.08 (obtained from sea bass), clustered with the RGNNV reference strains. The other six isolates, obtained from Senegalese sole and gilthead sea bream, were included within the SJNNV genotype (clade IV), although different subtypes could be recognized: subtype IVa contained one Spanish isolate (SpSs-IAusc1974.08), whereas the Japanese reference strains and the remaining Iberian isolates were included in subtype IVb (both groups clustering separately).

Restricting the analysis to a shorter fragment of both segments allowed the inclusion of 16 additional IBNNV sequences obtained from gilthead sea bream and Senegalese sole (GenBank accession nos GQ131547–GQ131571). The results obtained were identical to those described above: all RNA1 sequences clustered with the RGNNV genotype and all RNA2 sequences were included within the SJNNV genotype, except for SpDl-IAusc1688.08, which clustered with RGNNV (data not shown). In order to facilitate interpretation of the tree, the number of additional sequences was reduced to show those strains that exhibited differences with the remaining strains in either RNA1 (IAusc70.05) or RNA2 (IAusc347.05) and two strains representative of the 14 isolates showing identical sequences (Fig. 2).

ET analysis

Alignment of deduced amino acid sequences of the capsid protein from the six isolates showing an SJNNV-type RNA2 revealed the existence of five to six differences with the reference strains of this genotype (data not shown). Three of these differences were shared by the six isolates (positions 20, 247 and 270), which represented 0.9% of the amino acid composition of the capsid protein. However, comparison of the amino acid sequences of the RdRp (typed as RGNNV) with the reference strain SGWak97 showed 20–22 amino acid substitutions, 19 of them shared by all of the isolates (1.9% of the polymerase amino acid residues). Analysis of the isolate SpDl-IAusc1688.08, typed as RGNNV using both segments, revealed only three and seven differences in capsid and RdRp composition, respectively, when compared with the RGNNV type strain (data not shown).

Regarding the analysis of the 16 IBNNV sequences obtained directly from fish tissues, it was only possible to study a small region of both proteins. However, analysis of the capsid protein composition (aa 205–338) indicated that all of these isolates shared the differences with the reference strain at positions 247 and 270, already found in the strains that were completely sequenced. Unfortunately, the region of RdRp analysed in these isolates (aa 525–701) did not include any of the amino acid changes shared by the six isolates that were completely sequenced.

To determine whether the different residues observed in RdRp characterized the evolutionary process of the Iberian nodavirus, an ET algorithm was used. A phylogenetic tree was divided into ten partitions (I–X), sorting the sequences into different groups or classes, where each class consisted of a cluster of similar sequences originating from a given node within that partition. Two branches were observed in the first partition (Fig. 3): branch 1 contained reference strain SGWak97 and strain SpDl-IAusc1688.08, whereas branch 2 consisted of the remaining IBNNV sequences. Branch 2 was further subdivided in the following partitions in order of increasing divergence. Thus, partition IX...
Fig. 1. Phylogenetic relationships among seven Iberian betanodavirus isolates and reference strains from the four genotypes. (a) RNA1-based phylogeny (933 nt, nt 198–1130, with reference to SJNag93, GenBank accession no. AB056571). (b) RNA2-based phylogeny (836 nt, nt 186–1021, with reference to SJNag93, GenBank accession no. AB056572). Phylogeny was inferred using Mr Bayes 3.1 as implemented in TOPALi v2. Numbers indicate Bayesian PPs obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the first 50 000 generations. Sequences of two alphanodaviruses, BBV and NoV were used as an outgroup. Major betanodavirus groups are labelled according to Nishizawa et al. (1997) (*) and Thiéry et al. (2004) (†). Bars, 0.2 nucleotide substitutions per site.
Fig. 2. Phylogenetic analysis performed on a shorter fragment including 16 new sequences. (a) RNA1-based phylogeny (531 nt). (b) RNA2-based phylogeny (403 nt). In order to facilitate interpretation of the tree, the number of additional sequences was reduced to show those strains that exhibited differences with the remaining strains in either RNA1 (IAusc70.05) or RNA2 (IAusc347.05) and two strains representative of the 14 isolates showing identical sequences (IAusc08.06 and IAusc592.04). Phylogeny was inferred by the neighbour-joining method included in TOPALi v2. Two alphanodaviruses, BBV and NoV, were used as the outgroup. Bootstrap values are presented as percentages of 1000 resamplings. Major betanodavirus groups are labelled according to Nishizawa et al. (1997) (*) and Thiery et al. (2004) (†). Bars, 0.1 nucleotide substitutions per site.
distinguished SpSs-IAusc1974.08 from the other strains, and in partition X two subgroups were observed.

ET analysis of the six IBNNV strains showing an RGNNV-type RNA1 and an SJNNV-type RNA2 revealed that 14 aa substitutions observed in RdRp, from a total of 20–22, were class-specific (Fig. 4).

Predicted secondary structures of RdRp

In order to examine whether the 14 class-specific mutations could affect the structure of RdRp, secondary structure predictions were performed using two methods. Both methods agreed in the prediction and revealed only one change in the secondary structure (Fig. 5), affecting the ‘α–β–α’ arrangement between aa 258 and 299, by including an additional β-strand and reducing the length of the final α-helix. This change could be caused by some of the nearby point mutations: positions 218 (Phe→Leu, both non-polar and hydrophobic), 223 (Met→Leu, both non-polar and hydrophobic), 238 (Phe→Tyr, non-polar, hydrophobic→polar, hydrophilic) and 289 (Ala→Thr non-polar, hydrophilic→polar, hydrophilic).

Recombination analysis

The multiple approaches used to detect possible recombination events did not provide positive results. Although some possible recombination events were located by the MAXIMUM $\chi^2$ and CHIMAERA methods involving strain SpDI-IAusc1688.08, none of these events was found to be significant.

DISCUSSION

Betanodaviruses have been classified into four types, designated SJNNV, TPNNV, RGNNV and BFNNV, using a partial sequence of RNA2, the T4 region (Nishizawa et al., 1995, 1997). These types exhibit a different capability to infect fish species. Thus, RGNNV shows the broadest host range and causes disease in a variety of warm-water fish species, BFNNV is restricted to cold-water marine fish species and TPNNV infects only one species (Munday et al.,...
With regard to the SJNNV type, although for several years it was considered to be restricted to a few species present in Japanese waters (Nishizawa et al., 1997; Munday et al., 2002), in recent years it has been found in Senegalese sole (Thiéry et al., 2004; Cutrín et al., 2007), as well as gilthead sea bream and sea bass cultured in the Iberian Peninsula (Cutrín et al., 2007).

Phylogenetic analysis based on the full coding region of both genomic segments of the seven Iberian nodaviruses under study was intended to be performed including strains of the four established genotypes. However, no representative strains of the TPNNV genotype could be included, because, although sequencing of the complete genome of the type strain of this genotype has been reported (GenBank accession nos EU236148 and EU236149; Okinaka & Nakai, 2008), these sequences are not yet available in GenBank. Therefore, in order to include representative strains of the four genotypes, the phylogenetic analysis was performed on a shorter region of both segments. The phylograms obtained using both RNAs maintained the clusters corresponding to the different genotypes. However, six of the seven IBNNV strains exhibited markedly different genetic positions depending on which segment was analysed. The same results were obtained when phylograms were performed on complete coding region sequences in the absence of the TPNNV strain.

RNA2-derived phylogenies indicated that six strains clustered with the SJNNV reference strains, whereas only one, SpDI-1Ausc1688.08, was grouped with the RGNNV genotype. These results indicated that most of the strains belonged to the SJNNV type, as reported previously by our group in a more extensive study, which included typing (based on the T4 region) of 31 Iberian betanodaviruses (Cutrín et al., 2007). However, analysis of RNA1 depicted completely different phylogenetic relationships, and the seven isolates clustered with the RGNNV genotype. These results indicate a natural reassortment between strains of the SJNNV and RGNNV genotypes in six of the seven strains analysed. In addition, analysis of a shorter and different region of both segments indicated that the other 16 strains, seven of them previously classified as SJNNV type from an RNA2 analysis (Cutrín et al., 2007), are actually RGNNV/SJNNV reassortants.

Chimeric betanodaviruses using SJNNV and sevenband grouper nervous necrosis virus (belonging to RGNNV type) strains have been constructed previously in the

![Fig. 5. Secondary-structure predictions for the RdRp sequences of the IBNNV isolates (six reassortants and one RGNNV) and SGWak97 reference strain as provided by nps@ (Combet et al., 2000). Predicted α-helices of four or more residues are shown in blue, β-strands in red and random coils or turns as a horizontal purple line. The yellow-shaded area is expanded on the right.](image-url)
laboratory, thus demonstrating the feasibility of reassortment between both genotypes (Iwamoto et al., 2004). However, evidence of natural genetic reassortment occurring between different strains of betanodaviruses in a fish has been reported only twice (Névérez et al., 2005; Toffolo et al., 2007). Toffolo et al. (2007) reported that two strains from a group of 27 betanodavirus isolates harboured an RNA1 segment of SJNNV type and an RNA2 of RGNNV type. Both reassortant strains were obtained from symptomatic sea bass. These data indicated that both combinations of genomic segments of SJNNV and RGNNV genotypes are successful and allowed the resultant reassortant strains to produce disease. Interestingly, from the results of that study and the present one, a certain relationship between the type of reassortant and the susceptible host species seems to exist: SJNNV/RGNNV affecting sea bass and RGNNV/SJNNV affecting Senegalese sole and gilthead sea bream.

Genome segment reassortment is a complex phenomenon and there must be some requirement for the exchange to be effective. Analysis of the reassortant strains of the present study indicated differences in both genomic segments when compared with the reference strains of each genotype that could be involved in the success of the exchange process. Substitutions observed in the six strains represented 2.7 and 0.8% of the coding regions of RNA1 and RNA2, respectively. Analysis of the nucleotide mutations in the capsid protein ORF of the reassortant strains revealed that six of the eight changes (75%) observed in all strains rendered these SJNNV segments more similar to the RGNNV segment that they could have replaced. The same analysis performed on the RdRp ORF indicated that 34 of the 81 changes (42%) rendered these RGNNV segments more similar to the RNA1 of SJNNV type strain. If the acceptance of a heterologous segment depended on the number of mutations that rendered it more similar to the homologous one, we could speculate that our reassortant strains came from an RGNNV strain that accepted an RNA2 from an SJNNV strain. However, for some reovirus reassortants, it has been shown that only a few mutations are necessary for acceptance of a heterologous segment (Joklik & Roner, 1995). More recent results indicate that incorporation of an engineered ssRNA into the genome of reoviruses depends critically on three consecutive nucleotides of the 5′ untranslated region (UTR) (Roner et al., 2004). Unfortunately, we have not sequenced the complete UTRs of both genomic segments from all reassortant strains. Therefore, although we observed some nucleotide changes in these regions (data not shown), it is not possible to reach any reliable conclusion.

The total number of nucleotide mutations observed in genome segments of the reassortant strains resulted in 5–6 aa substitutions in the capsid protein (three shared by the six isolates) and 20–22 in the RdRp (19 shared by all of the isolates), which represented 0.9 and 1.9% of the respective sequences. This is remarkable, as structural proteins generally diverge more rapidly than non-structural ones, presumably in response to a greater environmental pressure.

Despite the changes in the amino acid composition of the reassortant RdRp (most of them described as class-specific by the ET analysis), most of the protein sequence revealed characteristic conservation patterns. In fact, all of the polymerase motifs identified in betanodaviruses (the acidic motif, aa 585–590; the SG.T motif, aa 646–651; the GDD motif, aa 686–688; the basic motif, aa 712; and an aromatic residue preceded by a basic sequence, aa 808) (Nagai & Nishizawa, 1999; Tan et al., 2001) were considered to be absolutely conserved regions in this analysis. In addition, as expected, the predicted secondary structure of RdRp did not reveal changes in the polymerase domain of the reassortant strains and the differences were restricted to the N-terminal one-third.

It has been reported that the capability of fish nodaviruses to infect different fish species is controlled by the coat protein (Iwamoto et al., 2004). In addition, it is known that even a small number of amino acid substitutions in the capsid proteins can have dramatic effects on the host specificity of different animal viruses (Baranowski et al., 2001). All of the reassortant strains exhibited a slightly modified SJNNV capsid, with three different amino acid positions in all strains (the differences increased to a maximum of six in some strains). One of these changes observed in residue 247 was encoded by the nucleotide triplet 737–739, included in the sequence nt 695–765, described by Ito et al. (2008) as a host-specific determinant. Another change in the amino acid sequence (aa 270) was also observed on the C-terminal side of the capsid protein. The existence of these two changes was also observed in the 16 reassortant isolates that were partially sequenced. These results confirmed that C-terminal protruding domains of the capsid protein are involved in host specificity, as reported previously (Iwamoto et al., 2004; Ito et al., 2008). However, the other change was observed in the N-terminal one-third of the protein. Tang et al. (2002) suggested that the N-terminal region from aa 1 to 82 of malabaricus grouper necrosis virus (belonging to the RGNNV genotype) may contribute to neutralization of the negative charge of the viral nucleic acid in virus assembly and RNA packaging. The significance of the mutations in this area in the reassortant strains needs to be clarified. The reassortant isolates obtained from Senegalese sole also shared the changes at aa 247 and 279, but showed additional differences at aa 293 or 298, depending on the strain. These results suggest that a few changes in the C-terminal domain of the capsid protein might favour the colonization of new hosts. Regarding this shift to new hosts, it is important to remember that all IBNNV sequences analysed in this study obtained from sea bream and Senegalese sole, including some previously classified as SJNNV, were reassortant strains.

The present study provides interesting insights into the possible evolution and colonization of new hosts by betanodavirus strains. The amino acid substitutions in
the capsid protein have possibly allowed broadening of the range of susceptible hosts for the SJNNV type. Further studies will be necessary to clarify the significance of the changes observed in the polymerase of the reассortant strains.

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