Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish

Toyohiko Nishizawa,1* Koh-ichiro Mori,1 Makoto Furuhashi,1 Toshihiro Nakai,1 Iwao Furusawa2 and Kiyokuni Muroga1

1Faculty of Applied Biological Science, Higashi-Hiroshima 724 and 2Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Striped jack nervous necrosis virus (SJNNV), a nodavirus, is the causative agent of viral nervous necrosis (VNN) in larval striped jack fish. In the present study, the SJNNV coat protein gene was sequenced and compared with that of four known insect nodaviruses and with four other fish nodaviruses causing VNN. The SJNNV coat protein gene was 1410 bases in length and contained a single ORF of 1023 bases encoding a protein of 340 amino acids. The sequence similarities between the coat protein gene of SJNNV and four insect nodaviruses were 28.6% or less at the nucleotide level and 10.6% or less at the amino acid level. A portion of the coat protein gene from four additional fish VNN viruses was amplified by PCR using primers designed for SJNNV and the amplified fragments (870–876 bases) were sequenced. The sequence similarities among SJNNV and the four VNN viruses were 75.6–84% or greater at the nucleotide level and 80.9% or greater at the amino acid level. In the fish nodaviruses a highly conserved region of 134 amino acids with sequence similarity of 92.5% or greater was detected. This conserved sequence was not found in the coat protein of insect nodaviruses. These results indicate that the fish nodaviruses that cause VNN are closely related to each other but are quite different from insect nodaviruses.

Introduction

Viral nervous necrosis (VNN) has been reported in hatchery-reared larvae and juveniles of marine fishes such as Japanese parrot fish (Oplegnathus fasciatus) (Yoshikoshi & Inoue, 1990), redspotted grouper (Epinephelus akaara) (Mori et al., 1991), striped jack (Pseudocaranx dentex) (Arimoto et al., 1993, 1994), Japanese flounder (Paralichthys olivaceus) (Nguyen et al., 1994), tiger puffer (Takifugu rubripes), kelp grouper (Epinephelus moara) (Nakai et al., 1994) and barfin flounder (Verasper moseri) in Japan since 1990. In other countries, a similar disease has also been reported in barramundi (Lates calcarifer) (Glazebrook et al., 1990; Munday et al., 1992), turbot (Scophthalmus maximus) (Bloch et al., 1991) and sea bass (Dicentrarchus labrax) (Breuil et al., 1991). These diseases cause high mortalities in hatchery-reared larvae and juveniles and are histopathologically characterized by vacuolation in the retinal and brain tissues. Unenveloped, round-shaped virions, approximately 25–30 nm in diameter, are always observed in the cytoplasm of the retinal and brain cells of infected fish.

Although the viruses causing these diseases cannot be grown using established fish cell lines, striped jack nervous necrosis virus (SJNNV), the causative agent of VNN in larvae of striped jack, has been purified from infected fish allowing the virological and molecular biological properties of the virus to be determined (Mori et al., 1992; Arimoto et al., 1993). The SJNNV genome consists of two single-stranded, positive-sense RNAs of 1.01 × 10⁶ Da (RNA1) and 0.49 × 10⁶ Da (RNA2). These RNAs have no poly(A) structure at the 3' terminus. RNA1 encodes a non-structural protein of about 100 kDa while RNA2 encodes a coat protein of about 42 kDa. On the basis of these properties, the SJNNV was identified as a new member of the family Nodaviridae. Although, apart from SJNNV, fish viruses causing VNN have not been characterized, a fluorescent antibody technique (FAT) using anti-SJNNV rabbit serum has revealed a common antigen among VNN agents of all fish species examined (Nguyen et al., 1994; Nakai et al., 1994). Recently, the aetiological agents of encephalitis in barramundi and sea bass were proven to be similar to SJNNV by analysis of their nucleic acids and coat proteins, and were identified as nodaviruses (Comps et al., 1994).

* Author for correspondence. Fax +81 824 22 7059.

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The Nodaviridae currently consists of one genus, Nodavirus, in which nodamura virus (NOV), black beetle virus (BBV), flock house virus (FHV) and boolarra virus (BOV) are listed (Francki et al., 1991). In the present study, the coat protein gene (RNA2) of SJNNV was cloned and sequenced in order to determine the relationship between SJNNV and the known insect nodaviruses (NOV, BBV, FHV and BOV). In addition, PCR was used to generate partial sequences of the coat protein gene of VNN viruses from four marine fish juveniles in order to compare the relatedness of fish nodaviruses including SJNNV.

**Methods**

**Virus source.** Diseased larvae and juveniles of striped jack, tiger puffer, barfin flounder, Japanese flounder and redspotted grouper were collected during outbreaks of VNN at several hatcheries in Japan and held at -80 °C for use in this study. All of these diseased fish were confirmed to have VNN by histopathological and electron microscopic examinations and by the FAT test using anti-SJNNV rabbit serum. The viruses in these affected fish were designated as TPNNV for tiger puffer, BFNNV for barfin flounder, JFNNV for Japanese flounder and RGNNV for redspotted grouper.

**Cloning and sequencing of SJNNV coat protein gene.** SJNNV was purified from diseased larvae of striped jack and the viral RNAs were extracted using the method described by Mori et al. (1992). The cDNAs were synthesized using a cDNA synthesis kit (Pharmacia). After addition of EcoRI adapters, the cDNAs were ligated into pBluescript (Stratagene) which was used to transform *Escherichia coli* DH5α (BRL). The recombinant plasmids containing cDNA inserts of 1-3-1.5 kbp were labelled with digoxygenin (Dig labelling kit, Boehringer Mannheim) for Northern hybridization with SJNNV RNAs. A series of unidirectional deletions of the cloned DNA were constructed and used for sequencing. Cloned cDNAs, which were synthesized with a PCR primer (R2) described below or with an oligo(dT) primer, were also used for sequencing of the 5′ and 3′ termini of the coat protein gene. Sequencing was performed using the Dye Primer Cycle Sequencing Kit (ABI), an automatic thermal cycler (Astec PC-700) programmed for one cycle at 72 °C for 10 min and 95 °C for 2 min, then 25 cycles at 95 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, and finally held at 75 °C for 5 min. PCR products were electrophoresed in 20% agarose-TAE buffer (40 mM-Tris-acetate, pH 7.2 and 1 mM-EDTA) gel, and stained with ethidium bromide.

**Cloning and sequencing of PCR products.** The sequences 5′-CCGA-ATTCGTGACG 3′ and 5′-CCGGGATACGCTCTT 3′ were added to the 5′ terminus of the forward primers (F1 and F2) or the reverse primer (R3), respectively. The former sequence contained EcoRI and SalI sites and the latter contained Smal, BamHI and HindIII sites. After digestion with EcoRI and BamHI, the PCR products were ligated into the pBluescript plasmid that was used to transform *E. coli* DH5α. Sequencing of the cloned PCR products was performed by the methods described.

**Computer analysis.** Sequences were assembled and analysed with MacDNASIS Pro (Hitachi Software Engineering) and MacVector (Eastman Kodak Company) computer programs.

**Results**

**Nucleotide and amino acid sequences of SJNNV coat protein gene.** The nucleotide sequence and deduced amino acid sequence of the SJNNV coat protein gene are shown in Fig. 2. The nucleotide sequence was 1410 bases long and contained a single ORF spanning positions 17-1039. Thus, there were 16 bases of 5′-non-coding region, and 371 bases of 3′-non-coding region. The ORF encoded a
Fish nodavirus coat protein genes

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the SJNNV coat protein gene.

<table>
<thead>
<tr>
<th>Amino acid similarity (%)</th>
<th>SJNNV</th>
<th>NOV</th>
<th>BBV</th>
<th>FHV</th>
<th>BOV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJNNV</td>
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<td>10.6</td>
<td>10.6</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>NOV</td>
<td>28.6</td>
<td>31.6</td>
<td>30.6</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>BBV</td>
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<td>41.7</td>
<td>86.7</td>
<td>39.6</td>
<td></td>
</tr>
<tr>
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<td>39.8</td>
<td>75.1</td>
<td>38.9</td>
<td></td>
</tr>
<tr>
<td>BOV</td>
<td>28.2</td>
<td>33.6</td>
<td>45.8</td>
<td>45.1</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Nucleotide and amino acid sequence similarities of the coat protein genes of SJNNV and known insect nodaviruses.

polypeptide of 340 amino acids with a calculated molecular mass of 37,180 Da. The sequence similarities between the coat protein gene of SJNNV and that of other known nodaviruses (NOV, BBV, FHV and BOV) (Dasgupta et al., 1984; Dasgupta & Sgro, 1989) were 28.6% or less at the nucleotide level and 10.6% or less at the amino acid level, while among the insect nodaviruses, NOV, BBV, FHV and BOV, the values were 33.6–73.1% at the nucleotide level and 20.3–86.7% at the amino acid level (Fig. 3). A Herr plot analysis revealed no significant
conserved regions of nucleotide or amino acid sequence between SJNNV and the other nodaviruses (data not shown).

RNA hybridization of coat protein genes of other VNN viruses

In all total nucleic acid preparations from diseased fish a target of about 1-4 kb was detected by Northern hybridization using a cDNA probe for the SJNNV coat protein gene (data not shown). The signal was strong in the SJNNV sample but relatively weak in the RGNNV, JFNNV, TPNNV and BFNNV samples.

PCR amplification of the coat protein gene of other VNN viruses

Portions of the coat protein gene of VNN viruses in the total nucleic acid extracts made from diseased fish with VNN were amplified using PCR primer sets for the T1–T5 regions of the SJNNV coat protein gene (Fig. 4). All regions of the SJNNV coat protein gene were amplified from the sample of infected striped jack and the resulting PCR products were approximately 1150 bp (T1:F1–R1 primers), 880 bp (T2:F1–R3 primers), 700 bp (T3:F2–R1 primers), 430 bp (T4:F2–R3 primers) and 180 bp (T5:F1–R2) coinciding with the predicted sizes. The PCR products corresponding to the T2 and T4 regions of SJNNV were also amplified from the coat protein gene of TPNNV, BFNNV, JFNNV and RGNNV using the total nucleic acid extracted from other fish species. No PCR products were produced using the primers for the T1, T3 or T5 regions.

Nucleotide and amino acid sequences of PCR products

The PCR products obtained from the T2 and T4 regions of the four additional VNN viruses were cloned and sequenced. The T4 region of each virus coincided with the 3' terminus of the T2 region. Multiple alignments of the nucleotide and the deduced amino acid sequences are shown in Fig. 5 and Fig. 6, respectively. The amplified PCR products of the T2 and T4 regions of TPNNV were same length as those of SJNNV, while BFNNV, JFNNV and RGNNV lacked 6 bases at positions 713–718 of the SJNNV coat protein gene as shown in the multiple alignment. Sequence similarities in the T2 regions of the five viruses were 75-8 % or greater at the nucleotide level and 80-9 % or greater at the amino acid level (Fig. 7a). The values for similarities between JFNNV and RGNNV were particularly high: 96-5 % nucleotide similarity and 97-5 % amino acid similarity. A highly conserved amino acid sequence with 92-5 % or greater similarity was noted at positions 83–216 among all of the viruses (Fig. 7b), and a relatively variable region with 65-4 % or less similarity was also noted at positions 235–315 (Fig. 7c).

Discussion

The nucleotide sequence of the SJNNV coat protein gene was 1410 bases long, which is almost the same size with the length of RNA2 estimated from agarose gel electrophoresis (Mori et al., 1992). The first AUG codon in the SJNNV coat protein gene occurs 17 bases from the 5' end. The minus three position of the AUG is an A and there is a G immediately following the AUG. This is consistent with a strong consensus for the eukaryotic ribosome binding model of Kozak (1981). Therefore, it was deduced that the ORF coding for the SJNNV coat protein is from the first AUG codon beginning at position 17 to the stop codon, UAA, beginning at position 1037. This ORF encodes a polypeptide of 340 amino acids with a calculated molecular mass of 37180 Da (Fig. 2). This value is about 5 kDa less than the apparent molecular mass of the SJNNV coat protein (42 kDa) as determined by SDS–PAGE (Mori et al., 1992).
In the RNA2 sequences of each of the four insect nodaviruses (NOV, BBV, FHV and BOV) there are 10–22 bases of 5'-non-coding region, 84–154 bases of 3'-non-coding region, two stem–loop structures at the 3' terminus, and a conserved sequence (5' CCCCUUAGCCGC 3') upstream of the stem–loop structures (Kaesberg...
et al., 1990). The sequence of the SJNNV RNA2, as determined in the present study, possessed non-coding regions of 16 and 371 bases in length at the 5' and 3' termini respectively, but neither stem loop structures nor the conserved sequence at the 3' terminus corresponding to those of NOV, BBV, FHV and BOV were detected. The leader sequence of the BBV coat protein gene is markedly rich in A (45–53%) while that of SJNNV contained only 31% A. The N-terminal structure of the BBV coat protein is very rich in basic amino acids and 16 of the first 50 amino acids are arginine (Dasgupta et al., 1984). The same feature was observed in the coat protein of NOV, FHV and BOV and was thought to be involved in the protein–RNA interaction necessary for encapsidation (Dasgupta et al., 1984). In the deduced amino acid sequence of the SJNNV coat protein, the N terminus is also very rich in basic amino acids with seven arginines and six lysines among the first 50 amino acids (Fig. 2). The sequence similarities between SJNNV and the four insect nodaviruses (NOV, BBV, FHV and BOV) are quite low and no conserved region is observed. On the basis of these results, it was concluded that SJNNV is quite different from the known insect nodaviruses in coat protein gene sequence.

The sequence data determined for TPNNV, BFNNV, JFNNV and RGNNV covered approximately 52% of the coat protein genes and about 82% of the amino acid sequence because these VNN viruses had an RNA2 almost the same size as that of SJNNV. This was considered to be a sufficient amount of data to discuss the genetic relationships among the fish VNN viruses and to compare them with the insect nodaviruses. The highly conserved 134 amino acid region (amino acids 83–216) of the coat protein in these VNN viruses appears to be specific to fish nodaviruses because it is not found in the coat protein sequence of known insect nodaviruses nor in any viral sequence in the GenBank database. Conversely, the 81 residues of the variable region (amino acids 235–315) of these fish nodaviruses are useful for distinguishing each virus genetically. Additionally, SJNNV and TPNNV can be distinguished from the other VNN viruses by the presence of the six bases at position 713–718 (Fig. 5) which are absent in BFNNV, JFNNV and RGNNV. SJNNV is also distinguishable from the other VNN viruses by the
production of PCR products representing the T1, T3 and T4 regions (Fig. 4). At positions 310–329 of the nucleotide sequence of BFNNV, JFNNV and RGNNV, there are sequences complementary to the reverse primer for the T5 region but no PCR product was amplified from the T5 region of these viruses. This might be due to subtle differences in the secondary structure around this region.

Our results indicate that these five fish nodaviruses are genetically distinguishable but closely related to each other. In particular, JFNNV and RGNNV are almost identical and share 95.1% sequence similarity, even in the variable region of their amino acid sequence (Fig. 7c). We suggest that these VNN viruses should not be placed within the known genus *Nodavirus* because of the great differences in overall sequence between these fish viruses and the known insect nodaviruses. We propose that a new genus be created in the family *Nodaviridae* to include the fish nodaviruses that cause VNN.

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References


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