

Genomic Classification of Fish Nodaviruses by Molecular Phylogenetic Analysis of the Coat Protein Gene

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A molecular phylogenetic analysis of 25 isolates of fish nodaviruses, the causative agents of viral nervous necrosis of marine fish, was performed based on the nucleotide sequences (427 bases) of the coat protein gene. These fish nodaviruses were classified into four clusters: tiger puffer nervous necrosis virus, striped jack nervous necrosis virus, berfin flounder nervous necrosis virus, and red-spotted grouper nervous necrosis virus.

The fish nodaviruses, new members of the family *Nodaviridae*, are the causative agents of viral nervous necrosis, or fish encephalitis, producing high mortalities in hatchery-reared larvae and juveniles of marine fishes in Japan, Southeast Asia, Australia, and Europe (1–3, 5, 8). Striped jack nervous necrosis virus (SJNNV), the causative agent of viral nervous necrosis in larvae of striped jack (*Pseudocaranx dentex*), was purified from heavily infected fish, allowing for virological and molecular biological analyses. SJNNV consists of a 42-kDa coat protein and two single-stranded, positive-sense RNAs, RNA1 and RNA2, without a poly(A) structure (7). RNA2 is 1,410 bases in length and contains a single open reading frame for a coat protein between nucleotide positions (nt) 17 and 1036. On the basis of the nucleotide sequence of RNA2, five different PCR primers were designed and applied to an analysis of coat protein genes of other fish nodaviruses (9, 10). A highly conserved

region with sequence identity of 93% or greater at the amino acid level and a variable region with sequence identity of 62% were observed on the coat protein genes of fish nodaviruses (9). In the present study, we compared the variable regions of the coat protein gene sequences of 25 isolates of fish nodaviruses in order to learn their molecular evolutionary relationships.

Sources of the fish nodavirus isolates used in this study are listed in Table 1. The isolates SJOri, TP93Kag, BF93Hok, JF93Hir, and RG91Tok correspond, respectively, to SJNNV, tiger puffer nervous necrosis virus (TPNNV), berfin flounder nervous necrosis virus (BFNNV), Japanese flounder nervous necrosis virus, and red-spotted grouper nervous necrosis virus (RGNNV), described in our previous paper (9). Extraction of total nucleic acids from affected fish and reverse transcription-PCR amplification of the viral gene were performed as previ-

TABLE 1. Sources of fish nodavirus isolates used in the present study

| Isolate | Source of isolate (scientific name) | Year | Country (prefecture) |
|----------|---|------|----------------------|
| SJOri | Striped jack (<i>Pseudocaranx dentex</i>) | 1991 | Japan (Nagasaki) |
| SJ91Nag | Striped jack (<i>Pseudocaranx dentex</i>) | 1991 | Japan (Nagasaki) |
| SJ92Nag | Striped jack (<i>Pseudocaranx dentex</i>) | 1992 | Japan (Nagasaki) |
| SJ93Nag | Striped jack (<i>Pseudocaranx dentex</i>) | 1993 | Japan (Nagasaki) |
| SJ94Nag | Striped jack (<i>Pseudocaranx dentex</i>) | 1994 | Japan (Nagasaki) |
| RS95Hir | Red sea bream (<i>Pagrus major</i>) | 1995 | Japan (Hiroshima) |
| TP93Kag | Tiger puffer (<i>Takifugu rubripes</i>) | 1993 | Japan (Kagawa) |
| JF95Hok | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1995 | Japan (Hokkaido) |
| BF93Hok | Berfin flounder (<i>Verasper moseri</i>) | 1993 | Japan (Hokkaido) |
| PC96Hok | Pacific cod (<i>Gadus macrocephalus</i>) | 1996 | Japan (Hokkaido) |
| RG91Tok | Red-spotted grouper (<i>Epinephelus akaara</i>) | 1991 | Japan (Tokushima) |
| RG94Oka | Red-spotted grouper (<i>Epinephelus akaara</i>) | 1994 | Japan (Okayama) |
| JF93Hir | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1993 | Japan (Hiroshima) |
| JF95Tok | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1995 | Japan (Tokushima) |
| JF94Wak | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1994 | Japan (Wakayama) |
| JF95Oit | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1995 | Japan (Oita) |
| JF95Sag | Japanese flounder (<i>Paralichthys olivaceus</i>) | 1995 | Japan (Saga) |
| SG94Oit | Seven-band grouper (<i>E. septemfasciatus</i>) | 1994 | Japan (Oita) |
| KG95Oit | Kelp grouper (<i>E. moara</i>) | 1995 | Japan (Oita) |
| PA94Oit | Purplish amberjack (<i>Seriola dumerili</i>) | 1994 | Japan (Oita) |
| JS95Shi | Japanese sea perch (<i>Lateolabrax japonicus</i>) | 1995 | Japan (Shizuoka) |
| MR93Tha | Malabar reef cod (<i>E. malabaricus</i>) | 1993 | Thailand |
| Sb95Ita | Sea bass (<i>Dicentrarchus labrax</i>) | 1995 | Italy |
| Omb95Ita | Ombrina (<i>Umbrina</i> sp.) | 1995 | Italy |
| Ba94Aus | Barramundi (<i>Lates calcarifer</i>) | 1994 | Australia |

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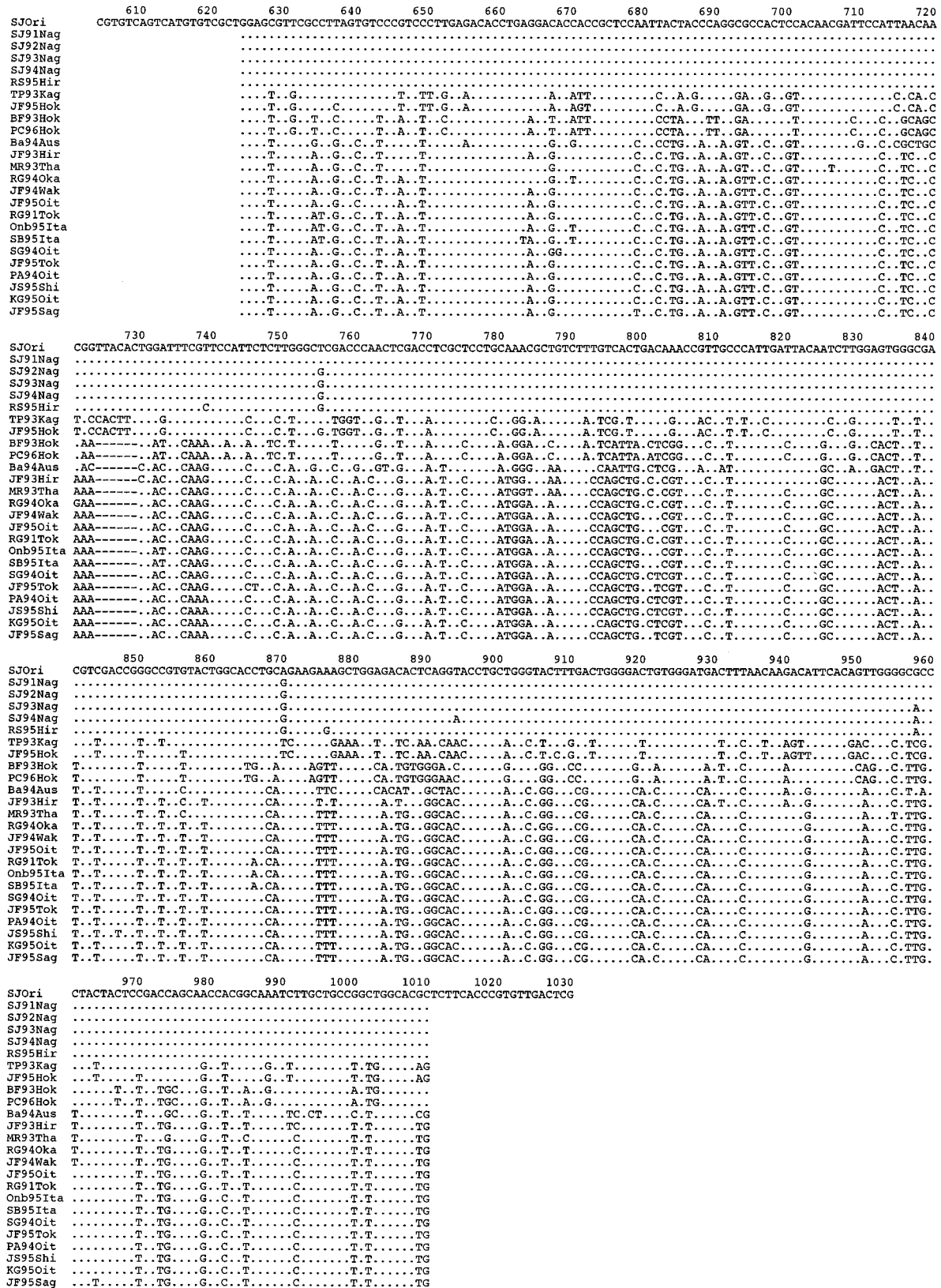


FIG. 1. Multiple alignment of determined nucleotide sequences of PCR products from the coat protein genes of 25 isolates of fish nodaviruses, nt 604 to 1030. This was constructed by the Clustal W program (12). Symbols: dot, nucleotide identical to that at the same position in the reference sequence of SJOri; hyphen, nucleotide gap inserted.

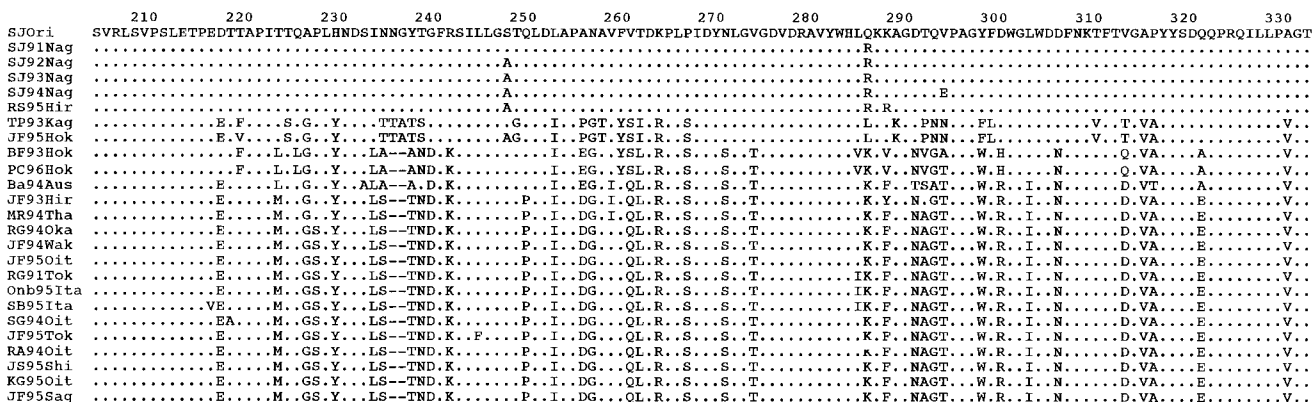


FIG. 2. Multiple alignment of deduced amino acid sequences of PCR products from 25 isolates of fish nodaviruses, constructed by the Clustal W program (12). Symbols: dot, nucleotide identical to that at the same position in the reference sequence of SJOri; hyphen, amino acid gap inserted.

ously described (9, 10). The PCR primers F2 (5'-CGTGTCA GTCATGTGTCGCT-3') and R3 (5'-CGAGTCAACACGGG TGAAGA-3') were used for the reverse transcription-PCR amplification of the target region of the viral genome, nt 604 to 1030, in which the variable region was included (9). The PCR products were cloned into the plasmid vector pCR-Script SK(+) (Stratagene) in order to determine the nucleotide sequence with a dye terminator cycle sequencing kit (Applied Biosystems [ABI]) and the auto sequencer A373-36 (ABI). Multiple alignments of determined nucleotide and deduced amino acid sequences were constructed with the Clustal W program (12), as shown in Fig. 1 and 2, respectively. The lengths of amplified PCR products from isolates SJ91Nag through -94Nag, RS95Hir, TP93Kag, and JF94Hok were the same as that of SJOri (SJNNV), while the other 17 isolates lacked 6 bases at positions corresponding to nt 724 to 729 of SJOri. Sequence identities of the PCR products among 25 isolates were 65.9% or greater at the nucleotide level and 68.0% or greater at the amino acid level.

The Dnaml program of PHYLIP 3.5c (4) was applied to search for an optimal tree with maximum likelihood criteria based on the multiple alignment of the nucleotide sequences. The transition/transversion ratio was set at 2.0, and 10 replicates of random-order taxon addition were performed by using the Jumble option. The final phylogenetic tree was drawn with the TreeView 1.0 program (11) and is shown in Fig. 3. The phylogenetic tree revealed that the Japanese isolates diverge into the following four major clusters: the TPNNV type, including TP93Kag and JF95Hok; the SJNNV type, including SJOri, SJ91Nag through -94Nag, and RS95Hir; the BFNNV type, including BF93Hok and PC96Hok; and the RGNNV type, including the other Japanese isolates. The sequence identities in each cluster were 95.0% or greater at both the nucleotide and amino acid levels even though the variable region was included as described above, indicating that each genotype is composed of several isolates of the same virus. Three foreign isolates, MR93Tha, Onb95Ita, and SB95Ita, were classified as belonging to the RGNNV type, with 95.0% or greater sequence identities among them. The one remaining foreign isolate, BA94Aus, showed 84.0% or greater sequence identities at the nucleotide and the amino acid levels with the RGNNV type.

The root of the present phylogenetic tree seems to exist at the point of divergence of the TPNNV-SJNNV types from the BFNNV-RGNNV types, because isolates of the BFNNV and RGNNV types lacked 6 bases at nt 724 to 729 and no typical

repeated sequence was observed peripheral to the missing sequences. By following the method of Li et al. (6), a molecular evolution rate of 2.6×10^{-3} nucleotide replacements/site/year was calculated based on the base substitution mutation rate of five SJNNV type isolates, which were obtained at the same place in five consecutive years from 1990 to 1994. Thus, the four major clusters of fish nodaviruses were considered to have diverged 100 to 150 years ago. The minor divergences in each cluster have occurred within the past 10 years, which is roughly consistent with the time when aquaculture operations, especially seed production in marine hatcheries, became prosperous.

It was interesting that Japanese isolates belonging to the RGNNV type were all located as progenies of Japanese flounder isolates in the phylogenetic tree. Seed production and culture of Japanese flounder are extensive in Japan, and juveniles are frequently transferred among fish farms over a wide area. Additionally, another Japanese flounder isolate, JF95Hok, is present in the TPNNV type, which is the type farthest genetically from the RGNNV type. Thus, Japanese flounder have

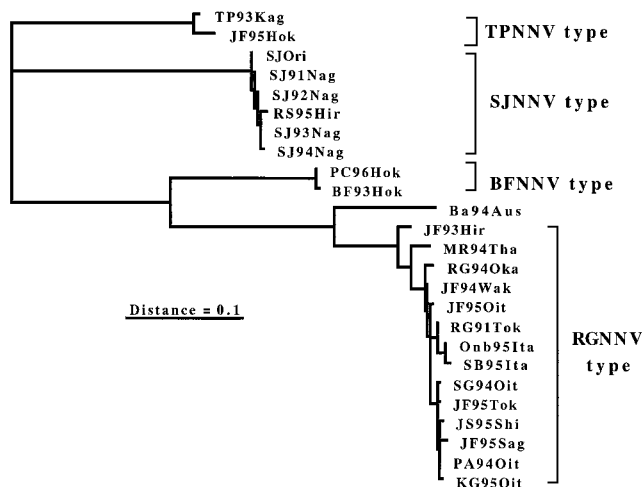


FIG. 3. Molecular phylogenetic tree deduced from analysis of the nucleotide sequences of 25 isolates of fish nodaviruses. The tree was built by the maximum likelihood criteria with the Dnaml program of PHYLIP 3.5c (4) and the TreeView 1.0 program (11). The lengths of horizontal branches are proportional to the numbers of nucleotide substitutions. Bar, 0.1 nucleotide replacement.

probably played a important role as carriers in the spread of fish nodaviruses in Japan.

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