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Phylogenetic analysis of betanodaviruses isolated from cultured fish in Korea

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ABSTRACT: Since the publication of the first report on fish nodaviruses in Korea in 1998 (Sohn et al. 1998; J Fish Pathol 11:97–104), fish nodaviruses have caused widespread epizootic events among various fish species in Korea. However, the genotypes of fish nodaviruses in Korea have not yet been determined due to a lack of information about their nucleotide sequences. In this study, we isolated 5 fish nodaviruses from 4 fish species cultured in 4 different regions in Korea: rock bream *Oplegnathus fasciatus*, Japanese flounder *Paralichthys olivaceus*, sevenband grouper *Epinephelus septemfasciatus*, and grey mullet *Mugil cephalus*. The full open-reading frame (ORF) encoding the coat protein (1017 nt) was sequenced from each of the 5 fish nodaviruses and the nucleotide sequences were phylogenetically analyzed. Results showed that even though their sequences were not identical, all 5 Korean isolates were clustered in the RGNNV genotype. This is the first report on the phylogenetic analysis of fish nodaviruses from cultured fish in Korea.

KEY WORDS: Fish nodavirus · Coat protein · Phylogenetic analysis

INTRODUCTION

Fish nodaviruses are the causative agents of viral nervous necrosis (VNN), also called viral encephalopathy and retinopathy (VER), in more than 30 fish species throughout the world (Munday et al. 2002), and cause mass mortality not only at early developmental stages but also at all other life stage (Fukuda et al. 1996, Tanaka et al. 1998). Fish nodaviruses were identified as a group of neuropathogenic viruses belonging to the family *Nodaviridae*, clearly distinguishable from the insect nodavirus *Alphanodavirus* (Tang et al. 2002), and recently classified in the genus *Betanodavirus* (Ball et al. 2000). The virus particles are spherical, non-enveloped and about 25 nm in diameter. They consist of a single coat protein and a bisegmented genome, RNA1 and RNA2 (Mori et al. 1992). The complete nucleotide sequences of RNA1 and RNA2 from several betanodaviruses have been reported, among them the striped jack nervous necrosis virus (SJNNV, the type species of the genus *Betanodavirus*) and the greasy grouper nervous necrosis virus (NNV) (Iwamoto et al. 2001, Tan et al. 2001). RNA1 (3.1 kb) encodes the viral replicase of about 100 kDa, while RNA2 (1.4 kb) encodes the coat protein of about 42 kDa (Mori et al. 1992, Nishizawa et al. 1995, Ball et al. 2000). Recently, a subgenomic transcript of RNA1, RNA3 (371 b), encoding a hypothetical B2 protein, has been detected in both the *Alphanodavirus* (Johnson et al. 2003) and *Betanodavirus* (Sommerset & Nerland 2004) genera.

Molecular phylogenetic analysis based on fish nodavirus nucleotide sequences (427 bases), including the variable region of the coat protein gene (aa 235–315) (Nishizawa et al. 1995), showed that fish nodaviruses were classified into 4 different genotypes: SJNNV; tiger puffer NNV (TPNNV); barfin flounder NNV (BFNNV); and red spotted grouper NNV (RGNNV) (Nishizawa et al. 1997). However, several reports postulate that there are betanodaviruses whose genotypes differ from those 4 genotypes. A betanodavirus isolate from the sea bass *Dicentrarchus labrax* farmed on the Atlantic coasts of France (Thiery et al.

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1999) was segregated as the earliest branch in the RGNNV genotype (Dalla Valle et al. 2001). In addition, Johansen et al. (2004) showed that the turbot nodavirus (TNV) is different from the 4 betanodavirus genotypes, supporting the possibility of a fifth genotype within the betanodaviruses.

In Korea, fish nodavirus was first reported in the sevenband grouper *Epinephelus septemfasciatus* by Sohn et al. (1998). Since then, there has been a high incidence of mortality among cultured fish in Korea caused by VNN. Five fish nodaviruses have been isolated from the following 4 fish species cultured in Korea: rock bream *Oplegnathus fasciatus*, Japanese flounder *Paralichthys olivaceus*, grey mullet *Mugil cephalus*, and sevenband grouper *E. septemfasciatus*. In the present study, we compared the nucleotide sequences of the coat protein of the 5 Korean fish nodavirus isolates to other available fish nodavirus sequences. Our results revealed that although there were sequence variations among the 5 isolates, they all belong to the RGNNV genotype.

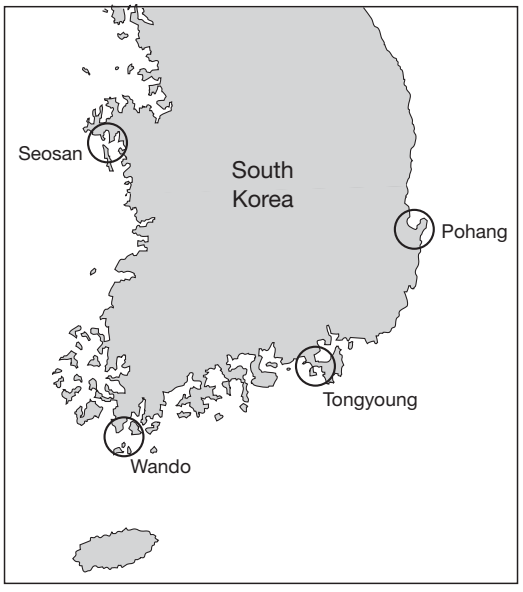

Fig. 1. Location of fish farms where epizootic events occurred and fish nodaviruses were isolated

MATERIALS AND METHODS

Virus samples. Between 2002 and 2004, epizootics occurred among rock bream *Oplegnathus fasciatus*, Japanese flounder *Paralichthys olivaceus*, grey mullet *Mugil cephalus*, and sevenband grouper *Epinephelus septemfasciatus* cultured in Korea (Fig. 1). Samples were collected from moribund fish. Sources of the 5 fish nodaviruses used in this study are listed in Table 1.

Total RNA was extracted from the fish samples' head regions, including the brain and eyes, using TRIZOL reagent (GIBCO-BRL). Five µg of DNase I-treated total RNA was reverse transcribed using random priming and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

Histology. Brain and retinal tissues of moribund fish were fixed in 10% neutralized buffered formalin (NBF), dehydrated, impregnated and embedded in paraffin wax. Serial 5 µm sections were collected on slide glasses coated with poly-L-lysine (poly-prep™ slides, SIGMA-ALDRICH) and stained with haematoxylin and eosin (H&E).

Polymerase chain reaction (PCR) of fish nodavirus genes. PCR was performed to amplify partial genes of fish nodavirus coat proteins. Primers for PCR were designed from nucleotide sequences in the GenBank/EMBL database of fish nodavirus coat proteins (AY324870). The PCR primers used are listed in Table 2. While Noda-partial primers were designed from a highly conserved region to detect most known fish nodavirus strains, Noda-full primers were designed to amplify the full open reading frame (ORF) of coat protein. The templates used were cDNA from the head regions of fish samples. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 35 cycles of 92°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. Three PCR products from each fish species were cloned into pGEM-T vector (Promega), and sequencing was performed at the Immunomodulation Research Center, in Ulsan, South Korea, on an automatic DNA sequencer (Applied Biosystems) according to the dye terminator procedure. Forward, reverse, and overlapping primers were designed from the sequencing results. The DNA

Table 1. Sources of fish nodavirus isolated from cultured fish in Korea

Isolate	Host species	Body size (cm)	Date	Geographic region
JFNNV-PH	Japanese flounder *Paralichthys olivaceus*	10	Jun 2002	Pohang
GMNNV-SS	Grey mullet *Mugil cephalus*	28	Jun 2002	Seosan
RBNNV-TY	Rock bream *Oplegnathus fasciatus*	10	Mar 2004	Tongyoung
SGNNV-TY	Sevenband grouper *Epinephelus septemfasciatus*	28	Mar 2004	Tongyoung
JFNNV-WD	Japanese flounder *Paralichthys olivaceus*	20	Oct 2004	Wando

Table 2. PCR primers used in gene amplification. Location on cDNA: primer location on cDNA of fish nodavirus coat protein (GenBank accession no. AY324870)

Primer	Sequence	Location on cDNA
Noda-partial-F	5′-CTGGGACACGCTGCTAGAAT-3′	300–319
Noda-partial-R	5′-CGACACGTTGACCACATCAG-3′	601–624
Noda-full-F	5′-TAATCCATCACCGCTTTGCAATCAC-3′	1–25
Noda-full-R	5′-TTCAAATTGGTCATCAACGATACGCACT-3′	1063–1090

and deduced amino acid sequences were compared with those in the GenBank/EMBL databases using BLAST.

***In situ* hybridization (ISH).** Digoxigenin (Dig)-labeled probes were prepared using PCR DIG Labeling Mix according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals). Briefly, PCR was performed with the reaction mixture of 100 µl containing 2 µM Noda-partial-F and Noda-partial-R primers (Table 2), 1.5 mM $MgCl_2$ (TAKARA), 5 U Taq polymerase (TAKARA), 10 µl PCR DIG Labeling Mix (Roche), 1 ng of plasmid construct of full ORF of coat protein. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 35 cycles of 92°C for 45 s, 60°C for 1 min, and 72°C for 2 min; and 1 cycle of 72°C for 5 min.

The tissue sections on slides were dewaxed and rehydrated by sequential immersion in xylene and graded alcohols. They were then treated with 10 µg ml^{-1} Proteinase K in Tris buffer (0.1 M, pH 8.0) for 30 min at 37°C. The slides were rinsed in Tris buffer for 3 min, dehydated in graded alcohols and dried in air. Hybridization mixture (50% formamide, 10% dextran sulphate, 4 × saline sodium citrate [SSC], 250 µg ml^{-1} yeast tRNA, 1 × Denhart's solution, 50 ng $µl^{-1}$ of DIG-labelled probe) was applied to the sections and incubated overnight at 42°C. The excess probe was removed and the sections were blocked using DIG wash and block buffer set (Roche). The slides were then incubated with anti-DIG antibody conjugated to alkaline phosphatase (Roche) for 1 h, and washed twice with DIG washing buffer (Roche) and rinsed in detection buffer (Roche) for 2 min. The color reaction was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt solution (BCIP/NBT solution, Roche) for 1 h in the dark. The excess substrate was removed by 3 washes of 2 min in 100 mM Tris-HCl buffer (pH 9.5) and the slides were counterstained with Bismarck brown Y, dehydrated, cleared and mounted using buffered glycerol.

Computer-assisted analysis. Sample sequences were aligned with those in the GenBank/EMBL databases using CLUSTAL W (Thompson et al. 1994). The phylogenetic tree was constructed with CLUSTAL W and then displayed with TreeView (Page 1996). Phylogenetic relationships among species were determined using the Neighbor-Joining method (NJ tree) (Saitou & Nei 1987). The reliability of the NJ tree was inferred using the Felsentein bootstrap method (Felsenstein 1985) with 1000 replicates.

The nucleotide sequence data reported in this paper was deposited in the GenBank database (GenBank accession numbers: JFNNV-PH, DQ116037; GMNNV-SS, DQ116038; RBNNV-TY, DQ116035; SGNNV-TY, DQ116036; JFNNV-WD, DQ864760).

RESULTS AND DISCUSSION

During 2002–2004, gross mortality occurred among rock bream, Japanese flounder, grey mullet, and sevenband grouper cultured in 4 different regions in Korea (Fig. 1, Table 1). The diseased fish were lethargic and a histopathological examination revealed vacuolation in brain tissue (Fig. 2A). These observations coincided with the characteristics of VNN. To determine whether or not the fish nodavirus was the causative agent of the epizootics, PCR was performed using Noda-Partial primers specific for the highly conserved region of coat protein of fish nodavirus designed from the nucleotide sequences in GenBank (AY324870) (Table 2). PCR products of the expected size (325 bp) were detected from the brain samples collected from the moribund fish (data not shown). ISH for betanodavirus showed strong positive signals within brain tissues. However, no significant signals were seen in kidney, liver and heart tissues (Fig. 2B–E). In order to determine the genetic characteristics of the fish nodavirus isolates, a full-length ORF of the coat protein of the fish nodavirus was amplified from 3 samples per fish species by PCR using Noda-full primers designed from the GenBank nucleotide sequence database of fish nodavirus (AY324870) (Table 2). The PCR products were cloned into a pGEM-T vector and the nucleotide sequence of the cloned DNA fragments was determined using M13 forward and M13 reverse sequencing primers in the vector and internal sequencing primers. Every nucleotide position in the coat protein gene was determined at least twice from each DNA strand. There was no difference in the nucleotide sequences of the coat protein full-length ORFs of the 3 fish nodavirus isolates (data not shown); 1 fish nodavirus isolate per species was used for further sequence analysis.

Sequence analysis of the coat protein full-length ORFs (1017 base [b] and 338 amino acids) revealed that the coat proteins shared 98 to 99% nucleotide

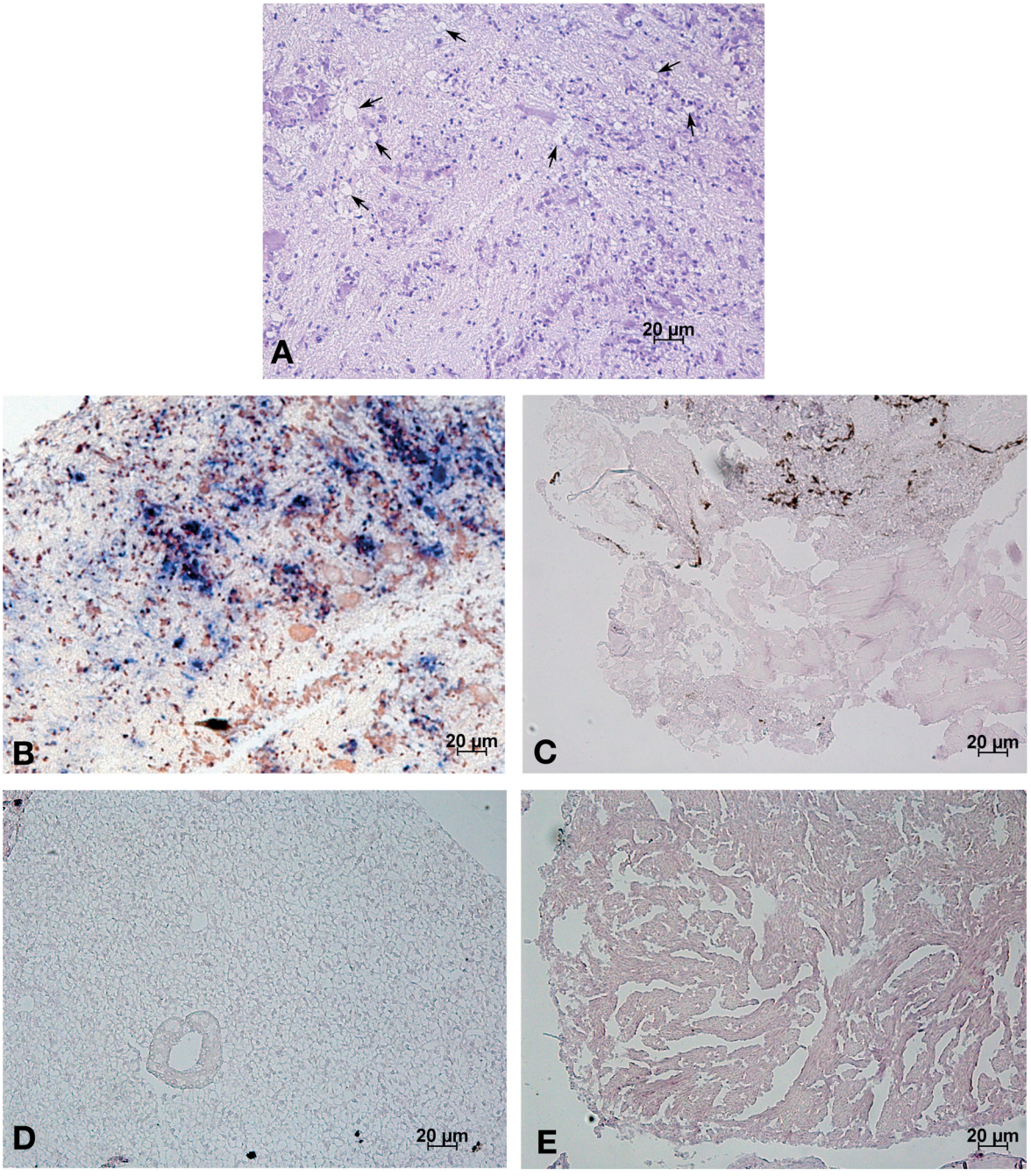

Fig. 2. *Paralichthys olivaceus.* Light microscopy of tissues. (A) Hematoxylin and eosin staining of brain tissues. Arrows indicate vacuoles in the brains. (B–E) *In situ* hybridization (ISH) for betanodavirus RNA. Strong ISH signals were observed in (B) brain tissues. No signals were detected in (C) kidney, (D) liver and (E) heart tissues. Black spots in kidney are melanin granules

sequence identity. They also shared 98 to 99% amino acid sequence identity and, except for 5 amino acid residues (22, 78, 282, 309, and 321), all other amino acid residues of the 5 Korean isolates were identical to each other (Fig. 3).

In order to determine the relationship between isolates of Korean fish nodaviruses and previously reported fish nodaviruses, we compared the nucleotide sequences of the coat protein ORFs from 5 Korean isolates to those of 39 other fish nodaviruses available in GenBank. As the first 168 nucleotides and the last 30 nucleotides did not correspond to sequences of many fish nodaviruses in GenBank, the analysis was restricted to nucleotides 169 to 987. This region

Fig. 3. Amino acid sequence alignment for the coat proteins of 5 Korean isolates. Letters with black background: identical residues across sequences; letters with grey background: conserved residues; letters with white background: residues without identity or similarity

includes the variable region (nucleotides 703 to 948) of fish nodavirus (Nishizawa et al. 1995), based on which fish nodaviruses were classified into 4 genotypes (Nishizawa et al. 1997). Phylogenetic analyses were carried out using the CLUSTAL W program with the default settings described by Johansen et al. (2004). In the phylogenetic tree, the fish nodaviruses used in the multiple alignments were divided into 5 groups (Fig. 4A). Among the 5 groups, 4 coincided with the genotypes TPNNV, SJNNV, BFNNV and RGNNV, as previously proposed by Nishizawa et al. (1997). Group 1, the RGNNV genotype, includes 33 fish nodavirus isolates from 21 fish species around the world. Group 2, the BFNNV genotype, includes 6 isolates that were obtained from cold-water species such as Atlantic halibut (AHNV), Atlantic cod (ACNNV), barfin flounder (BFNNV), haddock (HADNNV), and 1 isolate from sea bass in France (DIEV). Group 3, the SJNNV genotype, includes 3 isolates that were obtained from striped jack in Japan (SJNNV). Group 4, the TPNNV genotype, includes only 1 isolate, TPNNV, obtained from tiger puffer in Japan. However, Group 5 contained 1 fish nodavirus isolate, TNV, obtained from turbot in Norway, whose nucleotide sequences lay outside of the other 4 genotypes (Johansen et al. 2004). This suggests the possible presence of a fifth genotype of fish nodaviruses. All 5 Korean isolates fell within the RGNNV genotype, indicating that the members of the RGNNV genotype may be responsible for the epizootic episodes of nodavirus diseases in Korea.

RGNNV, the most widespread genotype, consisted of 33 isolates from 21 fish species around world, and strong evidence indicates that the RGNNV genotype should be further divided into 5 subgroups. Subgroups II, III, IV, and V each consisted of only 1 isolate: WGNNV from white grouper in Israel, WWNNV from white weakfish in Italy, JFNNV from Japanese flounder in Japan, and DIEV from European seabass in France, respectively. The remaining 29 isolates within the RGNNV genotype fell into Subgroup I. All 5 Korean isolates gathered in the Subgroup RGNNV I (Fig. 4B).

It is not clear when or from where the fish nodavirus was introduced into Korea. However, our results suggest that since the first detection of fish nodavirus in sevenband grouper in Korea (Sohn et al. 1998), fish nodaviruses have spread over a wide area. Transmission to various fish species in Korea has likely occurred through the frequent transfer of fish between fish farms. Because of the lack of information on nucleotide sequences, we could not determine the genotype of the first Korean isolate detected by Sohn et al. (1998). However, it is likely that the fish nodavirus detected by Sohn et al. (1998) also belonged to the RGNNV Subgroup I because all of the 5 currently prevailing isolates were identified as belonging to that subgroup.

Based on the coat protein gene, our phylogenetic tree, like previous observations (Nishizawa et al. 1997, Munday et al. 2002, Thiery et al. 2004), showed that host specificity of the RGNNV genotype is very low. Korean isolates from 4 different fish species showed a high similarity and all fell into the RGNNV Subgroup I. In addition, members of the RGNNV Subgroup I can infect at least 19 different fish species, and 2 different genotypes, BFNNV and RGNNV, can infect 1 fish species, the sea bass *Dicentrarchus labrax* (Fig. 4). It is

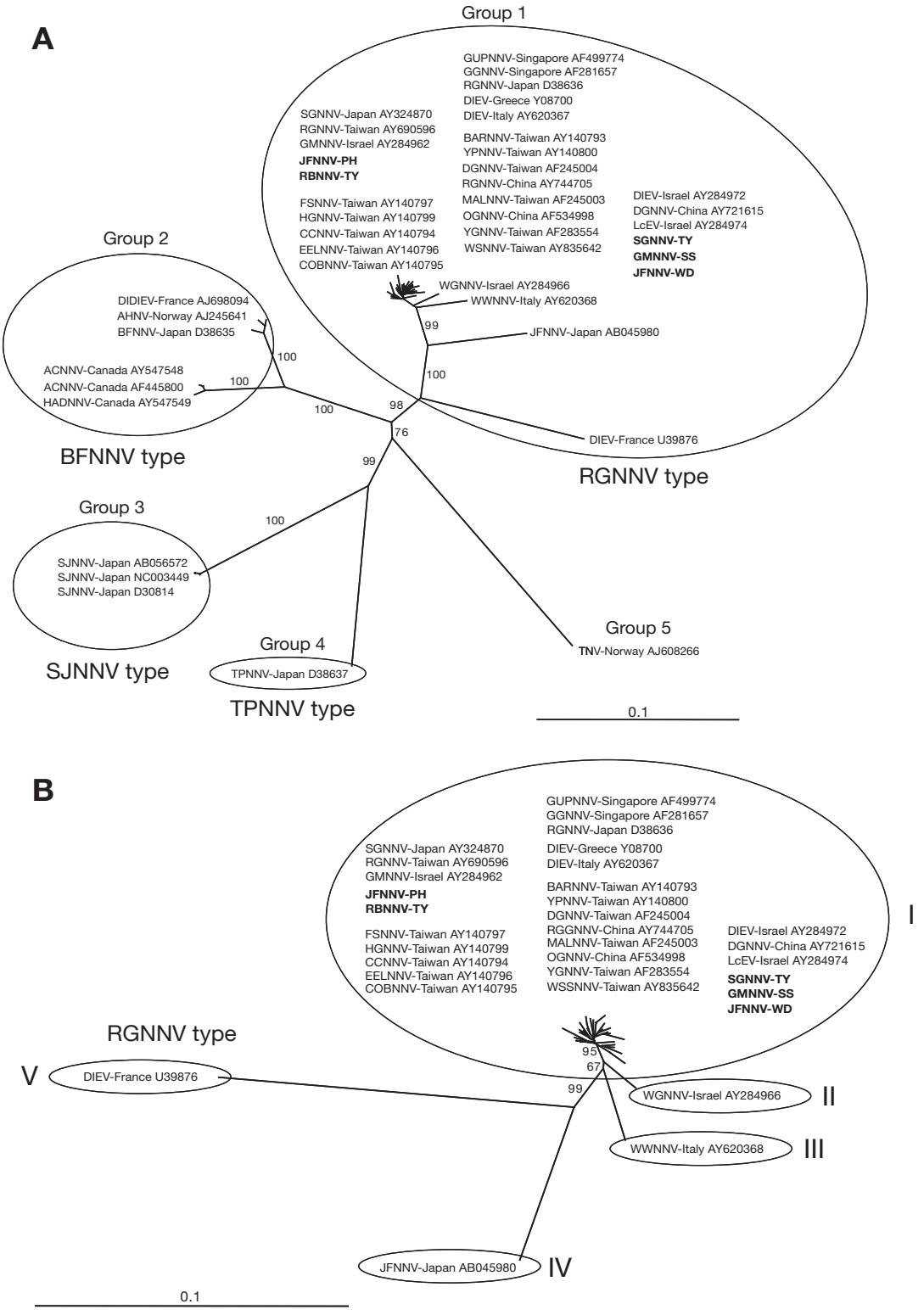

Fig. 4. Phylogenetic analysis of fish nodavirus coat protein. (A) Nucleotide sequences 169–987 from coat protein of 5 Korean fish nodavirus isolates (bold) were compared with those from 39 other fish nodaviruses. See 'Appendix 1' for GenBank accession numbers of nucleotide sequences and isolate abbreviations. (B) Phylogenetic positions of fish nodavirus isolates of Group 1, RGNNV genotype, from (A). GenBank accession numbers for nucleotide sequences are the same as in (A). Numbers indicate percent bootstrap support for each node from 1000 replicates. Distances are proportional to relative sequence deviations between individual nucleotide sequences. Phylogenetic analyses were carried out using CLUSTAL W. I–V: Subgroups I to V

uncertain whether modification of the coat protein gene is required for the nodavirus to adapt to another species of fish, or whether a slight modification (such as the changes seen in the 5 amino acid residues of the Korean isolates) is sufficient. Unlike the RGNNV genotype, the SJNNV and TPNNV genotypes have been isolated only from the striped jack *Pseudocaranx dentex* and the tiger puffer *Takifugu rubripes*, and there are several reports supporting the theory of host specificity among fish nodavirus strains. Cross-infection studies showed host specific pathogenicity among fish nodavirus strains (Thiery et al. 1999, Totland et al. 1999, Castric et al. 2001). Iwamoto et al. (2004) prepared reassortants between SGNNV, a member of the RGNNV genotype, and SJNNV, and found that the RNA2 encoding coat protein controls host specificity. Further study on the region or motif responsible for host specificity is necessary to better understand host specificity in fish nodaviruses.

Thiery et al. (2004) suggested a relationship between the grouping and geographical origin of nodaviruses. However, the phylogenetic position of the 5 Korean isolates was not related to geography in our study. The 5 isolates from different regions clustered into one subgroup (RGNNV I). Moreover, fish nodaviruses from 8 different countries clustered into the RGNNV Subgroup I. Frequent global trade of live fish could explain this observation (Hedrick 1996).

Our phylogenetic analysis supported the possible emergence of new genotypes besides the 4 main genotypes (TPNNV, SJNNV, BFNNV, and RGNNV) previously proposed by Nishizawa et al. (1997). One strain, TNV (AJ608266), from turbot in Norway, does not seem to belong to any of the 4 main genotypes (Johansen et al. 2004). In addition, even though we and Dalla Valle et al. (2001) placed JFNNV (AB045980, Japan) and DIEV (U39876, France) in the RGNNV genotype, these 2 isolates could be placed outside the RGNNV genotype with reasonable support from the data. In fact, Ucko et al. (2004) and Johansen et al. (2004) did place them outside the RGNNV genotype. Mori et al. (2003) reported a correlation between genotyping and serotyping of fish nodaviruses and have shown that genotypes SJNNV and TPNNV belong to Serotype A and B, respectively, while virus strains from RGNNV and BFNNV belong to Serotype C. It is possible that complete sequencing of RNA1 and RNA2 from an increasing number of fish nodaviruses in combination with their serological examination might reveal a new pattern for the genotyping of nodaviruses.

Here, we have phylogenetically analyzed 5 nodavirus isolates from 4 different fish species cultured in Korea. All 5 isolates fell within one subgroup, RGNNV I. The phylogenetic position of strain variants was not related to the host-dependent evolution and/or geography. Fish nodaviruses appear to adapt easily to other fish species. Thus, the prevention of further spread to other regions and other fish species cultured in Korea is urgent.

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Appendix 1. Fish nodaviruses and GenBank accession numbers for coat protein sequences in Fig. 4

Virus	Fish species	Country	Accession number
JFNNV-PH	Japanese flounder *Paralichthys olivaceus*	Korea	DQ116037
GMNNV-SS	Grey mullet *Mugil cephalus*	Korea	DQ116038
RBNNV-TY	Rockbream *Oplegnathus fasciatus*	Korea	DQ116035
SGNNV-TY	Sevenband grouper *Epinephelus septemfasciatus*	Korea	DQ116036
JFNNV-WD	Japanese flounder *Paralichthys olivaceus*	Korea	DQ864760
RGNNV	Redspotted grouper *Epinephelus akaara*	Japan	D38636
OGNNV	Orange-spotted grouper *Epinephelus coioides*	China	AF534998
DGNNV	Dragon grouper *Epinephelus lanceolatus*	China	AY721615
RGNNV	Redspotted grouper *Epinephelus akaara*	China	AY744705
DIEV	European seabass *Dicentrarchus labrax*	Israel	AY284972
DIEV	European seabass *Dicentrarchus labrax*	Greece	Y08700
DGNNV	Dragon grouper *Epinephelus lanceolatus*	Taiwan	AF245004
WSSNNV	White star snapper *Lutjanus stellatus*	Taiwan	AY835642
SGNNV	Sevenband grouper *Epinephelus septemfasciatus*	Japan	AY324870
RGNNV	Redspotted grouper *Epinephelus akaara*	Taiwan	AY690596
LcEV	Barramundi *Lates calcarifer*	Israel	AY284974
WGNNV	White grouper *Epinephelus aeneus*	Israel	AY284966
GMNNV	Grey mullet *Mugil cephalus*	Israel	AY284962
MALNNV	Malabaricus *Epinephelus malabaricus*	Taiwan	AF245003
GGNNV	Greasy grouper *Epinephelus tauvina*	Singapore	AF281657
DIEV	European seabass *Dicentrarchus labrax*	Italy	AY620367
GUPNNV	Guppy *Poecilia reticulata*	Singapore	AF499774
WWNNV	White weakfish *Atractoscion nobilis*	Italy	AY620368
YGNNV	Yellow grouper *Epinephelus awoara*	Taiwan	AF283554
DIEV	European seabass *Dicentrarchus labrax*	France	U39876
LcEV	Barramundi *Lates calcarifer*	Taiwan	AY140793
COBNNV	Cobia *Rachycentron canadum*	Taiwan	AY140795
EELNNV	European eel *Anguilla anguilla*	Taiwan	AY140796
CCNNV	Chinese catfish *Silurus asotus*	Taiwan	AY140794
HGNNV	Hump-back grouper *Cromileptes altivelis*	Taiwan	AY140799
JFNNV	Japanese flounder *Paralichthys olivaceus*	Japan	AB045980
ACNNV	Atlantic cod *Gadus morhua*	Canada	AY547548
HADNNV	Haddock *Melanogrammus aeglefinus*	Canada	AY547549
AHNV	Atlantic halibut *Hippoglossus hippoglossus*	Norway	AJ245641
SJNNV	Striped jack *Pseudocaranx dentex*	Japan	D30814
SJNNV	Striped jack *Pseudocaranx dentex*	Japan	NC_003449
SJNNV	Striped jack *Pseudocaranx dentex*	Japan	AB056572
TNV	Turbot *Scophthalmus maximus*	Norway	AJ608266
BFNNV	Barfin flounder *Verasper moseri*	Japan	D38635
DIEV	European seabass *Dicentrarchus labrax*	France	AJ698094
TPNV	Tiger puffer *Takifugu rubripes*	Japan	D38637
ACNNV	Atlantic cod *Gadus morhua*	Canada	AF445800
YPNNV	Yellow-wax pompano *Trachinotus falcatus*	Taiwan	AY140800
FSNNV	Firespot snapper *Lutaanus erythropterus*	Taiwan	AY140797

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