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Genomic classification of new betanodavirus isolates by phylogenetic analysis of the coat protein gene suggests a low host-fish species specificity

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Viral encephalopathy and retinopathy is a devastating disease that causes neurological disorders and high mortality in a large number of cultivated marine fish species around the world. It is now established that several viral strains classified in the genus *Betanodavirus* of the family *Nodaviridae* are the aetiological agents of this disease. Betanodaviruses can be classified into four genotypes based on the coat protein gene sequence. Here, the coat protein genes of the three major strains isolated from sea bass (*Dicentrarchus labrax*) in France were found to be different. In addition, 21 novel strains of betanodavirus from several fish species from France, Spain, Tunisia and Tahiti were classified by using phylogenetic analysis of a partial sequence (383 nt) of the coat protein gene. Most of the isolates were grouped in the red-spotted grouper nervous necrosis virus type, which was subdivided into two subtypes, one of them containing only French isolates. Furthermore, an isolate obtained from sea bass during an outbreak at low temperature (15 °C) was classified as the barfin flounder nervous necrosis virus type. This is the first reported isolation from sea bass of such a strain, which is known to infect several cold-water marine fish species. In addition, a betanodavirus belonging to the striped jack nervous necrosis virus type was detected in Senagalese sole (*Solea senegalensis*) farmed in Spain, which is the first indication of the presence of this genotype outside Japan. These findings suggest that the different genotypes can infect a variety of fish species and thus have a low host-fish species specificity.

INTRODUCTION

Betanodavirus is a recently recognized genus of the family *Nodaviridae*, which had previously been found only in insects (Ball *et al.*, 2000). Viruses belonging to this genus are the causative agent of viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis, a devastating disease of many species of marine fish cultured worldwide (Munday *et al.*, 2002). Affected fish commonly display neurological disorders, which are often associated with strong vacuolization of the central nervous system and the retina.

Betanodaviruses are small, spherical, non-enveloped viruses with a genome that is composed of two single-stranded, positive-sense RNA molecules. The larger genomic segment, RNA1 (3·1 kbp), encodes the RNA-dependent RNA polymerase (Chi *et al.*, 2001; Nagai & Nishizawa, 1999; Tan *et al.*, 2001), whilst the coat protein is encoded by RNA 2 (1·4 kbp) (Delsert *et al.*, 1997; Nishizawa *et al.*, 1994).

Comparison of the coat protein gene of five fish nodaviruses identified a highly conserved region (aa 83–216) and a variable region (aa 235–316) with amino acid sequence identities of 93 and 62 %, respectively (Nishizawa *et al.*, 1995). A classification of betanodaviruses based on comparison of the variable region of the coat protein gene among 25 isolates from farmed fish from Japan, Thailand, Italy and Australia was proposed by the same group (Nishizawa *et al.*, 1997). According to this, betanodaviruses could be classified into four types, designated striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and red-spotted grouper nervous necrosis virus (RGNNV). The same classification was subsequently used

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ698093–AJ698113.

by several groups. New fish nodavirus isolates from Europe and Asia were characterized and the majority of the isolates were classified as the RGNNV type (Skliris *et al.*, 2001). Another study of nodaviruses collected from fish farms in the Mediterranean basin has classified all isolates as the RGNNV type, whatever fish species they originated from (European sea bass, *Dicentrarchus labrax*, and shi drum, *Umbrina cirrosa*; Dalla Valle *et al.*, 2001). Recently, all betanodaviruses isolated from aquatic organisms in Taiwan have also been classified as the RGNNV type (Chi *et al.*, 2003). On the other hand, betanodaviruses isolated from cold-water fish species [e.g. Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) from Canada and the UK, and Dover sole (*Solea solea*)] were classified as the BFNNV type (Aspehaug *et al.*, 1999; Johnson *et al.*, 2002; Starkey *et al.*, 2000).

Previously, we reported that two isolates from European sea bass had distinct genomes (Thiéry *et al.*, 1999). The phylogenetic classification of one of these isolates, obtained from fish farmed on the Atlantic coast of France, was uncertain, as it segregated as the earliest branch in the RGNNV group in several studies (Aspehaug *et al.*, 1999; Dalla Valle *et al.*, 2001, R. Thiéry, unpublished observations).

In the present study, we have classified 21 new isolates obtained from various fish species farmed in France or the Mediterranean basin or from wild fish caught in Tahiti. Phylogenetic analysis of the variable region of the coat protein indicated that the new isolates could be classified into four main types or subtypes. For the first time, one isolate obtained from sea bass was classified as the BFNNV type, whereas an isolate from Senegalese sole (*Solea senagalensis*) farmed in Spain was classified as the SJNNV type, which was previously found only in Japan. Grouping of the isolates mainly correlated with their geographical origin rather than the fish species from which they were obtained, and a new classification nomenclature is suggested.

METHODS

Strains and virus cultivation. Betanodavirus isolates were obtained from diagnostic cases performed on various cultured marine fish species that were raised in different geographical areas (Table 1). Two isolates were obtained from wild fish caught in Tahiti lagoons and imported to France for ornamental fish trading. The SSN-1 cell line from *Channa striatus* (Frerichs *et al.*, 1996) was used for virus assay and propagation, as described previously (Castric *et al.*, 2001). After two passages, flasks were stored at −80 °C until further use.

RNA extraction. When needed, the contents of the flasks were thawed and total RNA was purified from 200 µl cell-culture supernatant by using a High Pure viral nucleic acid extraction kit (Roche) following the manufacturer's instructions. Some RNA purifications were also performed directly from fish-tissue extracts by using the same kit or a QIAamp viral RNA mini kit (Qiagen). Briefly, for small fish (<1 g), the entire heads were sampled and RNA was extracted as described above. For larger fish, RNA extractions were performed by using a pool of the brain and eyes of the fish. RNA was eluted in diethyl pyrocarbonate-treated water and kept frozen at −80 °C until analysis.

RT-PCR. Aliquots (3 µl) of total RNA samples were subjected to RT-PCR as described previously (Thiéry *et al.*, 1999), using primer sets that allowed amplification of the variable part of the coat protein gene, also referred to as the T4 region (Nishizawa *et al.*, 1994). Some RT-PCRs were also performed by using Ready-to-go RT-PCR beads (Amersham Biosciences) with an uninterrupted one-step protocol, following the manufacturer's instructions. After amplification, PCR products (420 bp) were analysed by electrophoresis on a 2 % agarose gel and stained with ethidium bromide. The entire coat protein gene sequences of two isolates (V26 and BB09) were also determined after cloning by using specific primers. For isolate V26, primers Vac1 and Vac2, which contain restriction sites, were used (Table 2). The amplified product was then cloned into pcDNAI (Invitrogen). For isolate BB09, several primers (Table 2) were designed according to the sequence of Atlantic halibut nodavirus (strain AH95NorA) (AH95NorA; GenBank accession no. AJ245641) and used to amplify different portions of the gene, which were cloned into pCRII-TOPO (Invitrogen).

Nucleotide sequencing and analysis. For strains V26 and BB09, plasmids containing the cloned PCR products were purified after propagation in *Escherichia coli* by using a QIAprep Spin Miniprep kit (Qiagen). Amplified products were purified by using a High Pure PCR products purification kit (Boehringer Mannheim). Plasmids and PCR products were sequenced in both orientations by using an ABI 373A automated sequencer (Perkin Elmer) and an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer), following the manufacturer's instructions. Alternatively, some PCR products were sent to Biotech companies for sequencing (GenomeExpress and MWG, France). Sequences were assembled by using DNASIS (Itachi).

After exclusion of the primer sequences, nucleotide sequences were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) or PILEUP as implemented in the GCG Wisconsin Package version 10.3 (Accelrys). The multiple nucleotide sequence alignment was inspected and finally edited manually by using GENEDOC (www.psc.edu/biomed/genedoc). Phylogenetic trees were inferred by several methods using PHYLOWIN (Galtier *et al.*, 1996) or the evolutionary programs implemented in GCG. Distance-based trees were constructed by using the neighbour-joining algorithm (Saitou & Nei, 1987) and 1000 bootstrap resamplings. Maximum-parsimony analysis was also performed by using the heuristic tree search option and 100 bootstrap resamplings with a PAUP search (GCG). Maximum likelihood analysis was performed by using a PAUP search with the nucleotide frequencies set to: fA = 0·22, fC = 0·303 and fG = 0·237; the transition/transversion ratio set to 1; and the following exchange rates: fAC = 1, fAG = 2·96, fAT = 1, fCG = 1 and fCT = 4·92. The trees were printed by using the TREEVIEW program (Page, 1996).

Mean similarities between the sequences of the betanodaviruses were determined by using the PLOTSIMILARITY program of the GCG Wisconsin Package version 10.3.

RESULTS

Genetic diversity of the coat protein of French betanodaviruses

Deduced amino acid sequences of the coat protein gene from the three major strains isolated from sea bass in France (V26, Y235 and BB09) were compared with available

Table 1. Origin of the studied betanodavirus isolates

Isolate	Isolation			Reference	GenBank accession no.
	Country (farm site code)	Year	Source of isolate		
Y235	France (a)	1991	Sea bass (*D. labrax*)	This report	AJ698105
V67	France (a)	1995	Sea bass (*D. labrax*)	This report	AJ698104
V26	France (b)	1996	Sea bass (*D. labrax*)	This report	AJ698093
V113	France (b)	1997	Sea bream (*Sparus aurata*)	This report	AJ698106
W80	France (b)	1998	Sea bass (*D. labrax*)	This report	AJ698107
W62	France (c)	1996	Sea bass (*D. labrax*)	This report	AJ698098
V68	France (c)	1997	Sea bass (*D. labrax*)	This report	AJ698099
X82	France (c)	1999	Sea bass (*D. labrax*)	This report	AJ698095
X130	France (c)	1999	Meagre (*Argyrosomus regius*)	This report	AJ698108
X199	France (c)	1999	Meagre (*Argyrosomus regius*)	This report	AJ698103
Y55	France (c)	2000	Sea bass (*D. labrax*)	This report	AJ698102
Y154	France (c)	2000	Meagre (*A. regius*)	This report	AJ698096
X137	France (d)	1999	Sea bass (*D. labrax*)	This report	AJ698100
Y193	France (d)	2000	Sea bass (*D. labrax*)	This report	AJ698097
X149	France (e)	1999	Sea bass (*D. labrax*)	This report	AJ698101
BB09	France (f)	2003	Sea bass (*D. labrax*)	This report	AJ698094
Sp 20/Sba	Spain	1998	Sea bass (*D. labrax*)	Skliris *et al.* (2001)	AF175515
03-160	Spain	2003	Senegalese sole (*Solea senegalensis*)	This report	AJ698113
It/23/Sba	Italy	1995	Sea bass (*D. labrax*)	Skliris *et al.* (2001)	AF175513
It/19/Sba	Italy	1997	Sea bass (*D. labrax*)	Skliris *et al.* (2001)	AF175514
DlEV	Greece	1996	Sea bass (*D. labrax*)	Sideris (1997)	Y08700
Mt/01/Sba	Malta	1995	Sea bass (*D. labrax*)	Skliris *et al.* (2001)	AF175512
V82	Tunisia (g)	1992	Sea bass (*D. labrax*)	This report	AJ698109
BB155	Tunisia (g)	2003	Sea bass (*D. labrax*)	This report	AJ698110
V9954	Scotland	1999	Halibut (*H. hippoglossus*)	Starkey *et al.* (2000)	Not available
AH95NorA	Norway	1995	Halibut (*H. hippoglossus*)	Grotmol *et al.* (2000)	AJ245641
AHNNV	Norway	1995	Halibut (*H. hippoglossus*)	Aspehaug *et al.* (1999)	AF160473
Z233	Tahiti	2001	Convict surgeonfish (*Acanthurus triostegus*)	This report	AJ698111
Z243	Tahiti	2001	Narrowstripe cardinalfish (*Apogon exostigma*)	This report	AJ698112
Sjori	Japan	1991	Striped jack (*Pseudocaranx dentex*)	Nishizawa *et al.* (1995)	D30814
RG91Tok	Japan	1991	Red-spotted grouper (*Epinephelus akaara*)	Nishizawa *et al.* (1995)	D38636
JF93Hir	Japan	1993	Japanese flounder (*Paralichthys oliveatus*)	Nishizawa *et al.* (1995)	D38527
BF93Hok	Japan	1993	Barfin flounder (*Verasper moseri*)	Nishizawa *et al.* (1995)	D38635
Jp/15/Rp	Japan	1994	Rock porgy (*Oplegnatus punctatus*)	Skliris *et al.* (2001)	AF175520
Jp/06/Rp	Japan	1995	Striped jack (*Pseudocaranx dentex*)	Skliris *et al.* (2001)	AF175519
Tp93Kag	Japan	1995	Tiger puffer (*Takifugu rubripes*)	Nishizawa *et al.* (1995)	D38637
ETNNV	Singapore	1991	Greasy grouper (*Epinephelus tauvina*)	Tan *et al.* (2001)	AF281657
SG/14/Bar	Singapore	1995	Barramundi (*L. calcarifer*)	Skliris *et al.* (2001)	AF175516
GNNV	Singapore	2000	Guppy (*P. reticulata*)	Hegde *et al.* (2003)	AF499774
MGNNV	Taiwan	Unknown	Malabar grouper (*Epinephelus malabaricus*)	Lin *et al.* (2001)	AF245003
DGNNV	Taiwan	Unknown	Giant grouper (*Epinephelus lanceolatus*)	Lin *et al.* (2001)	AF245004
YGNNV	Taiwan	2000	Yellow grouper (*Epinephelus awoara*)	Lai *et al.* (2001)	AF283554
Th/07/Bgr	Thailand	1995	Brown-spotted grouper (*Epinephelus chlorostigma*)	Skliris *et al.* (2001)	AF175518
ACNNV	Canada	1999	Atlantic cod (*G. morhua*)	Johnson *et al.* (2002)	AF445800

betanodavirus coat protein sequences from different countries and hosts: SJNNV (GenBank accession no. D30814) from Japan, dragon grouper nervous necrosis virus (DGNNV) (AF245004) and malabaricus grouper nervous necrosis virus (MGNNV) (AF245003), both from Taiwan, *D. labrax* encephalitis virus (DlEV) (Y08700) from Greece and AH95NorA (AJ245641) from Norway. As the first 17 nt of the coat protein ORF of AH95NorA are lacking in the corresponding reported sequence (AJ245641), the analysis was restricted to aa 7–338. By using the programs PILEUP and DISTANCE (GCG), which compute the pairwise percentage substitution for 100 aa, we found the sequence

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5′→3′)	Use
Vac1*	TACAAGCTTATGGTACGCAAAGGTGAT	V26 RNA2 cDNA cloning
Vac2†	TACTCTAGATTAGTTTTCCGAGTCAAC	V26 RNA2 cDNA cloning
412RNA2 F2	GACCAATGACGTCCATCTC	BB09 RNA2 cDNA (5′ end) cloning
412RNA2 R2	TGACCCGGTTAGTTTCCCG	BB09 RNA2 cDNA (5′ end) cloning
412RNA2 F3	CGACCACAAAGGCCGTTAA	BB09 RNA2 cDNA (central region) cloning
412RNA2 R4	AGTTGGATCAGGCAGGAAG	BB09 RNA2 cDNA (central region) cloning
412RNA2 F6	TGATGTCGACCGTGCCGTT	BB09 RNA2 cDNA (3′ end) cloning
412RNA2 R6	CGGTAACCCAACAAGCCCA	BB09 RNA2 cDNA (3′ end) cloning

**Hin*dIII restriction site is underlined.

†*Xba*I restriction site is underlined.

identities presented in Table 3. Interestingly, strain V26 was highly related to DlEV (100 %), also isolated from sea bass, thus confirming our previous observation (Thiéry *et al.*, 1999) that was made by using a smaller region of the coat protein gene, but strain BB09 was more highly related to strain AH95NorA (99 %), isolated from halibut, than to DlEV (86 %) or SJNNV (81 %). The coat protein sequence of strain Y235 was only partially related to the other fish betanodavirus coat protein sequences: DlEV (88 %), AH95NorA (84 %) and SJNNV (81 %), which also confirmed our previous observations (Thiéry *et al.*, 1999).

The sequences of other nodaviruses were included in the analysis, such as the coat protein precursor α of several alphanodaviruses from insects [Flock house virus (FHV), GenBank accession no. X15959; black beetle virus (BBV), X00956; Boolarra virus (BoV), X15960; Nodamura virus (NoV), X15961; Pariacoto virus (PaV), AF171943] and the recently reported sequence of the coat protein of an unclassified nodavirus, *Macrobrachium rosenbergii* nodavirus (MrNV, AY222840; Sri Widada *et al.*, 2003), isolated from the giant freshwater prawn, *M. rosenbergii*. These sequences were only distantly related to the fish betanodavirus sequences (between 10 and 15 % amino acid identities). Nevertheless, by using the powerful block search program MACAW, we found a stretch of basic-rich residues, close to the N-terminus of the coat protein, that was conserved among all nodaviruses (Fig. 1). The phylogeny among nodaviruses was inferred tentatively by using the aligned coat protein sequences and both neighbour-joining and maximum-parsimony analyses. The alphanodaviruses and betanodaviruses segregated clearly into different clades, with significant support from bootstrap values using both methods (Fig. 2). Compared with each other, the betanodaviruses were more homologous (80–100 % identity) than the alphanodaviruses (40–60 % identity) and the branch-lengths of the betanodavirus subtree were significantly shorter than those of the alphanodaviruses. The studied betanodaviruses segregated into three major branches: SJNNV type, DlEV type and AH95NorA type. The DlEV branch was subdivided into two sub-branches that were supported by significant bootstrap values. Although strains Y235, DlEV and BB09 were isolated from the same fish species (but from different geographical origins and on different dates), they clearly belonged to different genotypes or subgenotypes.

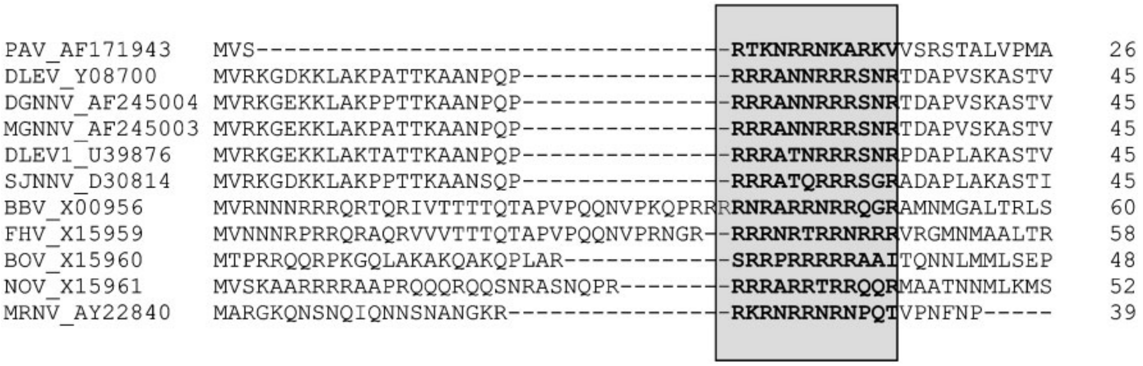
Table 3. Identities (%) of the deduced amino acid sequences of the coat protein gene of three strains isolated in France compared with published nodavirus sequences

Isolate	SJNNV	DNNV	MGNNV	DlEV	AH95NorA
V26	82	99	99	100	87
Y235	81	89	89	88	84
BB09	81	85	85	86	99

Phylogenetic relationships among betanodavirus isolates from various geographical locations

The level of genetic heterogeneity of the coat protein genes of the betanodaviruses was analysed by using the PLOTSIMILARITY algorithm with a 10 nt sliding window (Fig. 3). One region was found to depict a higher level of heterogeneity and corresponded to the T4 genomic region described previously (Nishizawa *et al.*, 1995). As this region is particularly well-suited to distinguishing the different betanodavirus genotypes, we determined the T4 nucleotide sequence of all isolates. After comparison of our sequences to related sequences from GenBank (Table 1) by multiple sequence alignment, the phylogenetic relationships among all sequences were assessed (Fig. 4).

Five main clusters that were supported by significant bootstrap values were obtained. Cluster Ia included several isolates that were obtained from different fish species and sites in France, Tunisia and Tahiti. The sequences of these isolates were highly related to sequences obtained from betanodavirus strains obtained from fish suffering the



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PAV_AF171943     MVS----------------------------------RTKNRRNKARKVVSRSTALVPMA     26
DLEV_Y08700      MVRKGDKKLAKPATTKAANPQP---------------RRRANNRRRSNRTDAPVSKASTV     45
DGNNV_AF245004   MVRKGEKKLAKPPTTKAANPQP---------------RRRANNRRRSNRTDAPVSKASTV     45
MGNNV_AF245003   MVRKGEKKLAKPATTKAANPQP---------------RRRANNRRRSNRTDAPVSKASTV     45
DLEV1_U39876     MVRKGEKKLAKTATTKAANPQP---------------RRRATNRRRSNRPDAPLAKASTV     45
SJNNV_D30814     MVRKGDKKLAKPPTTKAANSQP---------------RRRATQRRRSGRADAPLAKASTI     45
BBV_X00956       MVRNNNRRRQRTQRIVTTTTQTAPVPQQNVPKQPRRRRNARRNRRQGRAMNMGALTRLS     60
FHV_X15959       MVNNNRPRRQRAQRVVVTTTQTAPVPQQNVPRNGR--RRRNRTRRNRRRVRGMNMAALTR     58
BOV_X15960       MTPRRQQRPKGQLAKAKQAKQPLAR------------SRRPRRRRRAAITQNNLMMLSEP     48
NOV_X15961       MVSKAARRRRAAPRQQQRQQSNRASNQPR--------RRRARRTRRQQRMAATNNMLKMS     52
MRNV_AY22840     MARGKQNSNQIQNNSNANGKR----------------RKRNRRNRNPQTVPNFNP-----     39
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Fig. 1. Conserved region among nodavirus coat proteins. Sequences were compared by using MACAW, which finds blocks of conserved amino acids. A stretch of basic-rich amino acids was found to be conserved at the N-terminus of the coat protein among all the nodaviruses. The shaded block had a highly significant similarity score ($P<0{\cdot}001$) among the different nodaviruses. The position of the last aligned amino acid residue in each sequence is indicated on the right. Gaps (indicated by '-') have been inserted to allow alignment of the sequences.

disease at temperatures above 25 °C, such as sea bass from Greece and Italy or grouper species from south-east Asia and Japan that were reported previously to belong to the RGNNV type. The nucleotide sequences of isolates W80 (sea bass) and V113 (sea bream) were identical to that of isolate V26 (sea bass) and were all obtained from fish raised at the same site. Strain V113 was isolated from asymptomatic sea bream (Castric *et al.*, 2001). Interestingly, strains obtained from wild fish in Tahiti and cultured sea bass in Tunisia formed a subtype within cluster Ia that was supported by a high bootstrap value. Other isolates from cluster Ia tended to segregate in sub-branches according to their geographical origin, but the bootstrap values were low.

Cluster Ib contained French isolates only. Strain Y235 is the same strain that was reported previously as DlEV (Delsert *et al.*, 1997), SBNNV-Atl (Thiéry *et al.*, 1999) and SB1 (Breuil *et al.*, 2001) and was obtained in 1991 from sea bass that was cultured on the Atlantic coast of France. Another isolate obtained 3 years later in the same farm was highly

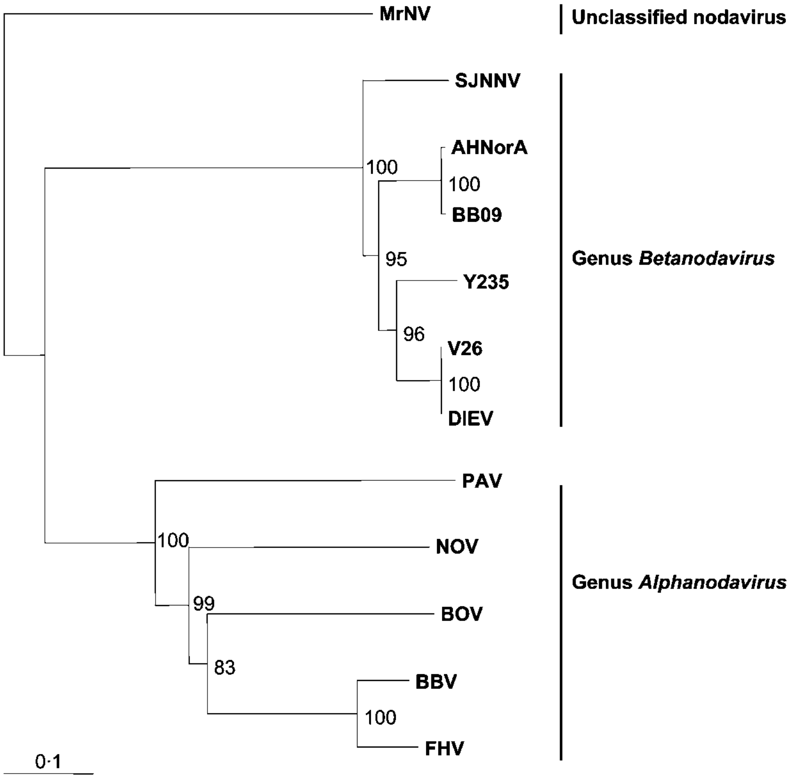

Fig. 2. Phylogeny among nodaviruses. The deduced amino acid sequences of the coat protein precursor gene of several alphanodaviruses and the coat protein of various betanodaviruses were aligned by using PILEUP. Phylogeny was inferred by using the neighbour-joining method included in PHYLOWIN. Bootstrap values are indicated as percentages of 1000 resamplings. The MrNV coat protein sequence (AY222840) was used as the outgroup.

related (V67). Strains X199 and Y55 were obtained from meagre and sea bass, respectively, grown on a different farm. Several isolates had the same nucleotide sequence as Y55 (X82, X137, X149, V68, W62, Y154 and Y193) and were either obtained from the same farm or from different farms that exchanged live fish.

Cluster II contained several isolates that were obtained from cold-water species: Atlantic halibut (Norway and Scotland), Atlantic cod (Canada) and barfin flounder (Japan). Surprisingly, strain BB09 obtained from sea bass in France during a VER outbreak that occurred at a temperature of about 15 °C and was associated with high mortality also clustered into this genotype. To our knowledge, this is the first description of such a nodavirus in European sea bass. Several subtypes were clearly defined within cluster II and correlated with the geographical origin of the isolates. Subtype IIa contained the Atlantic cod strain from Canada (AF445800). Subtype IIb contained a barfin flounder isolate from Japan. Subtype IIc contained European isolates that were obtained from different fish species. These subtypes

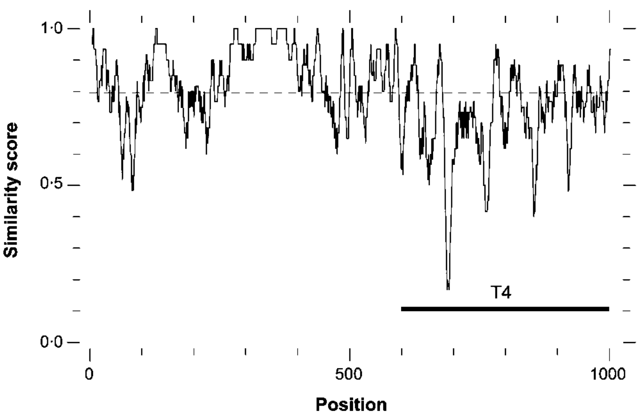

Fig. 3. Similarity plot along the coat protein gene of four betanodaviruses. The PLOTSIMILARITY program was used to draw similarity score curves by means of a 10 nt sliding window using four betanodavirus type strains: Sjori (D30814), AH95NorA (AJ245641), Y235 (U39876) and DIEV (Y08700). Bar, position of the T4 region according to Nishizawa *et al.* (1995).

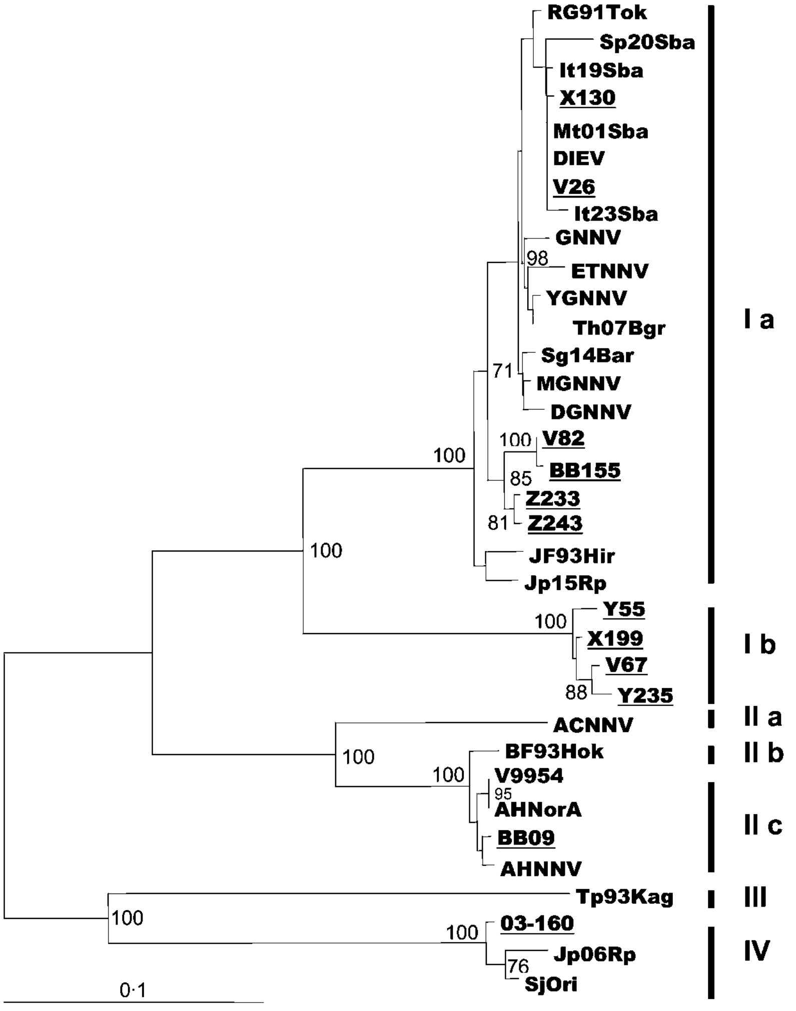

Fig. 4. Unrooted phylogenetic tree of the studied betanodavirus isolates. The nucleotide sequences of the T4 region (383 nt) of all isolates from this study (underlined) and related isolates available in GenBank were aligned by using PILEUP. Phylogeny was inferred by using the neighbour-joining method included in PHYLOWIN. Bootstrap values are indicated as percentages of 1000 resamplings.

were supported by high bootstrap values. Cluster II corresponded to the BFNNV type (Nishizawa *et al.*, 1997).

Cluster III was represented here by a single isolate, TPNNV, obtained from tiger puffer in Japan and is the type species of the TPNNV type (Nishizawa *et al.*, 1997).

Cluster IV (SJNNV type) contained strains that were isolated from Japanese striped jack (Nishizawa *et al.*, 1997; Skliris *et al.*, 2001). Surprisingly, strain 03-160, obtained from Senegalese sole (Spain), also clustered within this genotype and is the first strain belonging to this genotype to be found in Europe. This strain was associated with mortality at 30 °C.

DISCUSSION

In the present study, phylogeny among nodaviruses was inferred by using deduced amino acid sequences from the coat protein gene of three distinct betanodavirus strains isolated in France and other available sequences from alpha- and betanodaviruses. Results strongly support the accepted classification (Ball *et al.*, 2000) of these viruses into two distinct genera, as sequences from insect nodaviruses (genus *Alphanodavirus*) and fish nodaviruses (genus *Betanodavirus*) grouped into two separate clades. The sequence of a newly reported nodavirus isolated from giant freshwater prawn (Sri Widada *et al.*, 2003) could not be assigned to either of the existing genera and could thus represent a novel genus within the family *Nodaviridae*. It should be noted that these results were obtained by using deduced amino acid sequences and that we could not infer phylogeny by using the corresponding nucleotide sequences, as they were too divergent to obtain correct alignments (data not shown). Nevertheless, by using the conserved block search program MACAW, we found a stretch of basic-rich residues close to the N-terminus of the coat protein that is highly conserved in all known nodaviruses. It is very likely that this region interacts with the coated viral RNA, as suggested previously (Tang *et al.*, 2002).

The phylogeny among 21 new betanodavirus isolates was subsequently studied by using a smaller region of the coat protein gene, which was shown to be highly variable, in agreement with Nishizawa *et al.* (1995). Comparison of the sequences of these isolates with other sequences available in GenBank and phylogenetic analysis indicated that they clustered into four types or subtypes. Previously, we reported that farmed sea bass could be infected by two genetically distinct betanodavirus strains (Thiéry *et al.*, 1999). One of these strains was also isolated from asymptomatic sea bream, whereas it was associated with mortality in sea bass (Castric *et al.*, 2001). In the present work, a third genetically distinct strain (BB09) causing mortality in sea bass at low temperature was characterized. It clustered into a distinct group (II), which contained isolates that were shown to induce the disease in cold-water fish species in several geographical areas (Japan, Norway, the UK and Canada). It was related closely to the other European isolates from this cluster described so far, here designated IIc. Although all strains from cluster II have a common ancestor, the distinct subtypes correlated strongly with their geographical origins, indicating that they subsequently evolved independently. Fish infected by strain BB09 were the progeny of brood stock that was caught in the wild with no history of the disease at higher temperatures. They suffered from encephalopathy and retinopathy when a sudden drop in temperature occurred accidentally in the premises. As they were not in contact with other farmed cold-water species, the infection presumably occurred in the wild in the brood stock and was subsequently transmitted vertically. This would concur with the observation that vertical transmission of the disease can occur in sea bass at low temperature (15 °C; Breuil *et al.*, 2002).

A strain infecting farmed Senegalese sole in Spain (in the Mediterranean Sea) was detected and assigned to cluster IV (SJNNV). This is the first description of this genotype in Europe, as it has only been described previously in farmed fish from Japan: striped jack and red sea bream (Nishizawa *et al.*, 1997). According to the Fish Base (www.fishbase.com), the natural area of distribution of Senegalese sole ranges from the Bay of Biscay to the coastal waters of Senegal and the Canary Islands; thus, there is a risk of dissemination of this strain to other farmed fish species. However, the sequence of this virus was distinct from the virus sequences from Japan, suggesting that a different strain belonging to this genotype circulates in eastern Atlantic waters. It should be noticed that the Senegalese sole sample was considered to be nodavirus-free by using a cell-culture assay (Frerichs *et al.*, 1996) followed by an indirect fluorescent-antibody test (IFAT) using a polyclonal antibody raised against sea bass isolates (J. Castric, personal communication). Thus, the evidence of a nodavirus infection came from the sequence of PCR products obtained after amplification of RNA samples extracted from fish tissues. The reason why no virus could be detected using cell culture and IFAT is not completely understood. It is likely that the virus was present in high quantities, as mortality was severe. It was reported that IFAT using a rabbit polyclonal serum raised against SJNNV was able to detect nodavirus in sections of virus-infected barramundi larvae (Munday *et al.*, 1994), demonstrating some antigenic similarity between the two viruses. However, the same serum used in ELISA is only specific for SJNNV (OIE, 1995) and several nodavirus isolates were neutralized by a polyclonal antibody raised against a nodavirus isolated from sea bass with the exception of one isolate obtained from striped jack (Skliris *et al.*, 2001). Taken together, these data suggest that antigenic differences among nodavirus strains may influence antibody recognition in diagnostic procedures.

Most of the isolates clustered into group I, previously designated RGNNV. However, this cluster could be divided into two subtypes with good support values. It was particularly interesting that subtype Ib only contained isolates that were obtained from fish species raised in France.

These strains were obtained from three different fish species (sea bass, shi drum and meagre), which suggested that this subtype may also spread to several fish species. Before this study, only one strain belonging to this subtype had been described (Delsert *et al.*, 1997; Thiéry *et al.*, 1999), originating from a sea bass farm located on the French Atlantic coast and corresponding to strain Y235 described here. Thus, we have now provided evidence that this subtype is distributed among several French farms. Previous phylogenetic studies including this strain have classified it into the RGNNV type (Aspehaug *et al.*, 1999; Dalla Valle *et al.*, 2001), as have we. Interestingly, strains V26 and Y235, belonging to clusters Ia and Ib, respectively, showed distinct pathogenicity to sea bass larvae (Breuil *et al.*, 2001). Moreover, strain Y235 could be transmitted from experimentally infected sea bass females to the eggs and larvae, whereas strain V26 could not (Breuil *et al.*, 2002). The different biological properties probably rely on different temperature optima needed for replication. Thus, it seems that there are sufficient lines of evidence to classify these strains into different subtypes. Whether subtype Ib should be considered as a new genotype depends on the level of identity that is required to define a genotype, which varies considerably from one virus family to another.

The rest of the isolates clustered into group Ia and tended to segregate on different sub-branches according to their geographical origin, although bootstrap values were low. A noticeable exception was that strains from Tunisia grouped with strains obtained from wild fish species caught in Tahiti. Four isolates from France (V26, W80, V113 and X130) were in the same subgroup as those obtained from sea bass in Spain, Italy and Greece. All of these strains were highly related to strain RGNNV (98 % nucleotide sequence identities), as reported previously (Nishizawa *et al.*, 1997; Sideris, 1997; Thiéry *et al.*, 1999). The sequence of three isolates obtained from two different fish species from the same site were identical (V26, W80 and V113), but different from that of strain X130, which was obtained from another species (meagre) and another site. Interestingly, strain X130 belonged to a different subtype from the other isolates found in the same site, which all gathered in cluster Ib. These data suggested that at least two different viral introductions occurred at the farm. All other isolates from cluster Ia were obtained in fish species raised in countries from south-east Asia: grouper species from Taiwan (Lin *et al.*, 2001) and Singapore (Hegde *et al.*, 2002), barramundi (*Lates calcarifer*) from Thailand (Skliris *et al.*, 2001) and the recently reported guppy nervous necrosis virus (GNNV) strain from guppy (*Poecilia reticulata*) in Singapore (Hegde *et al.*, 2003).

Overall, the topology of the phylogenetic trees obtained in this study was similar to those obtained in previous studies on betanodaviruses using varying lengths of nucleotide sequences from the same gene (Dalla Valle *et al.*, 2001; Nishizawa *et al.*, 1997; Skliris *et al.*, 2001). Thus, the T4 coat protein gene region (Nishizawa *et al.*, 1997) is sufficiently informative to assess phylogenetic relationships among betanodaviruses. Nevertheless, it should be noted that several isolates that were obtained at an interval of several years from fish grown at the same site had the same sequence. This suggests that the coat protein gene has a low evolutionary rate. On the other hand, some isolates obtained from different farm sites shared the same T4 nucleotide sequence: Y55 (site c), X137 and Y193 (site d), and X149 (site e). Commercial exchange between these sites could explain this observation. Thus, comparison of the T4 region of the coat protein gene between isolates could also provide an interesting epidemiological tool to investigate the origin of an infection.

At present, all reported phylogenetic studies on betanodaviruses have used sequences derived from the coat protein gene. The genome of nodaviruses is composed of two single-stranded RNAs that encode the RNA-dependent RNA polymerase and the coat protein. Available sequences derived from the RNA polymerase gene are still scarce (Nagai & Nishizawa, 1999; Tan *et al.*, 2001), but further characterization of this gene for an increasing number of isolates will provide complementary data to assess the evolutionary relationships among betanodaviruses in greater detail.

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