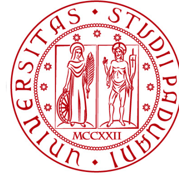

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A MULTIDISCIPLINARY APPROACH TO THE STUDY OF BETANODAVIRUSES IN THE MEDITERRANEAN BASIN

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RIASSUNTO

I virus appartenenti alla famiglia *Nodaviridae*, genere *Betanodavirus* sono gli agenti eziologici della necrosi nervosa virale (NNV), una delle patologie ittiche economicamente più gravose per l'acquacoltura marina. La NNV è diffusa in tutto il mondo, in particolare nelle aree caratterizzate dalla presenza di allevamenti intensivi. Nel bacino del Mediterraneo dove la malattia è endemica, la NNV è stata recentemente riconosciuta come uno dei principali fattori limitanti per lo sviluppo dell'acquacoltura marina e, allo stesso tempo, come una minaccia per la fauna selvatica. Nonostante l'importanza ecologica ed economica di questa patologia, ad oggi le conoscenze sulle proprietà biologiche di betanodavirus sono ancora limitate, e le strategie di controllo attualmente utilizzate sono basate esclusivamente sulla diagnosi precoce e sull'applicazione di misure di biosicurezza.

Questa tesi si focalizza sullo studio della NNV nel bacino del Mediterraneo, e si prepone di investigare le caratteristiche genetiche, fenotipiche ed ecologiche dei genotipi di betanodavirus circolanti in questo areale, con particolare interesse verso i riassortanti recentemente descritti. I dati generati nella presente tesi contribuiscono allo sviluppo di opportune strategie di controllo per limitare la diffusione dell'infezione e forniscono conoscenze di base per lo sviluppo futuro di vaccini contro la NNV.

SUMMARY

Viruses belonging to the family *Nodaviridae*, genus *Betanodavirus* are the causative agents of viral nervous necrosis (VNN), one of the most devastating and economically relevant diseases for marine aquaculture. VNN is spread worldwide, particularly in densely farmed areas. In the Mediterranean basin the disease is endemic, and has recently been recognized as the major limiting factor for the development of marine aquaculture, besides being a threat for wild fish stocks. Despite the ecological and economical importance of VNN, limited knowledge is available on the biological features of betanodaviruses, and current control strategies are only based on early diagnosis and on the adoption of biosafety measures.

The present thesis focuses on the study of VNN in the Mediterranean region, and on a careful investigation of the genetic, phenotypic and ecological features of betanodavirus genotypes currently circulating in this area, with particular attention to the newly emerged reassortants. In addition, data generated in this thesis contributes to the development of control strategies to limit the spread of the infection and provides some basic knowledge for future development of vaccines protective against VNN.

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Preface

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Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe

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A B S T R A C T

Viral encephalopathy and retinopathy (VER) is one of the most devastating diseases for marine aquaculture, and similarly represents a threat to wild fish populations because of its high infectivity and broad host range. Betanodavirus, the causative agent of VER, is a small non-enveloped virus with a bipartite RNA genome comprising the RNA1 and RNA2 segments. We partially sequenced both RNA1 and RNA2 from 120 viral strains isolated from 2000 to 2009 in six different countries in Southern Europe. Phylogenetic analysis revealed the presence of the red-spotted grouper nervous necrosis virus (RGNNV) ($n = 96$) and striped jack nervous necrosis virus (SJNNV) ($n = 1$) genotypes in Southern Europe, with 23/120 samples classified as RGNNV/SJNNV reassortants. Viruses sampled from individual countries tended to cluster together suggesting a major geographic subdivision among betanodaviruses, although some phylogenetic evidence for viral gene flow was also obtained. Rates of nucleotide substitution were similar to those observed in a broad array of RNA viruses, and revealed a significantly higher evolutionary rate in the polymerase compared to the coat protein gene. This may reflect temperature adaptation of betanodaviruses, although a site-specific analysis of selection pressures identified relatively few selected sites in either gene. Overall, our analyses yielded novel data on the evolutionary dynamics and phylogeography of betanodaviruses and therein provides a more complete understanding of the distribution and evolution of different genotypes in Southern Europe.

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1. Introduction

Viral encephalopathy and retinopathy (VER) is an highly infective neuropathological disease that affects a broad spectrum of fish species, and often results in fatal outcomes particularly in larval and juvenile stages (Munday et al., 2002). Several papers report episodes of mass mortality caused by VER outbreaks with a significant economic impact on marine aquaculture (Glazebrook et al., 1990; Grotmol et al., 1995; Le Breton et al., 1997; Munday et al., 1992; Ransangan and Manin, 2010).

The causative agent of VER is a small, non-enveloped virus of the family *Nodaviridae* within genus *Betanodavirus*. Its genome consists of two single stranded positive-sense RNA molecules, RNA1 (3.1 Kb) and RNA2 (1.4 Kb), which encode the RNA-dependent RNA polymerase (RdRp) and the capsid protein (CP), respectively (Mori et al., 1992). A subgenomic transcript called RNA3, which originates from the 3′ terminus of RNA1 during virus replication, allows the accumulation of viral RNA into the host cell (Fenner et al., 2006; Iwamoto et al., 2005). Based on phylogenetic analysis of the T4 variable region within the RNA2 segment, betanodaviruses have been historically divided into four genotypes, namely striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). Although Thiéry et al. (2004) proposed a different nomenclature system (Clusters I–IV), many authors agreed with the original taxonomic classification which has been used in several papers for genotyping purposes (Chi et al., 2003; Gomez et al., 2008a, 2009; Grotmol et al., 2000; Hegde et al., 2003; Johnson et al., 2002; Moody et al., 2009). Characterization of partial RNA2 sequences from new betanodavirus isolates led to the suggestion of two additional genotypes: the Atlantic

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cod nervous necrosis virus (ACNNV) (Gagné et al., 2004) and the turbot nodavirus (TNV) (Johansen et al., 2004).

Interestingly, Iwamoto et al. (2000) demonstrated that distinct genotypic variants possess specific optimal culture temperatures *in vitro*: 25–30 °C for RGNNV, 20–25 °C for SJNNV, 20 °C for TPNNV and 15–20 °C for BFNNV. In agreement with these observations, which correlate viral clustering with specific environmental conditions, it has been reported that different betanodavirus genotypes have distinct geographic distributions and, accordingly, distinct host-ranges, as a result of an adaptation to different water temperatures (Chérif et al., 2009). For example, BFNNV appeared to affect cold-water fish species in North America, Norway and Japan as well as sea bass (*Dicentrarchus labrax*) in France (Grotmol et al., 2000; Johnson et al., 2002; Nishizawa et al., 1997; Nylund et al., 2008; Thiéry et al., 2004), while TPNNV was isolated from *Takifugu rubripes* in Kagawa, Japan (Nishizawa et al., 1997). Similarly, SJNNV was originally considered to be restricted to Japanese waters, but was later detected in fish reared in Spain and Portugal (Cutrín et al., 2007; García-Rosado et al., 2007; Thiéry et al., 2004). Of all the betanodaviruses, the RGNNV genotype has the broadest range of warm-water hosts and has the widest geographic distribution throughout Asia, USA, Australia (Munday et al., 2002) and the Mediterranean basin (Chérif et al., 2009; Ciulli et al., 2006, 2007; Dalla Valle et al., 2001; Skliris et al., 2001; Thiéry et al., 2004). Interestingly, Lopez-Jimena et al. (2010) demonstrated in specimens of wild asymptomatic meagre (*Argyrosomus regius*) the coexistence of both SJNNV and RGNNV genotypes, which might favor the occurrence of genetic reassortment events. The presence of reassortant betanodaviruses has been previously described in sea bass caught in Italy and Croatia, in the form of a genetic variant containing the RNA1 segment deriving from the SJNNV genotype and the RNA2 molecule originating from the RGNNV-type (Toffolo et al., 2007). Later on, a new reassorted betanodavirus, in the form of a RGNNV/SJNNV genetic variant, has been detected in sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) farmed in Portugal and Spain (Olveira et al., 2009).

The clear importance of betanodaviruses for marine aquaculture, as well as their genetic diversity, highlights the need to further investigate the molecular evolution and epidemiology of VER in Southern Europe. To that end we investigated the evolutionary dynamics and phylogeography of betanodaviruses in this area by analyzing the RNA1 and RNA2 gene segments of 120 viral isolates collected between 2000 and 2009 from six European countries.

2. Materials and methods

2.1. Samples

Out of a collection of 335 isolates, we selected 120 viral strains gathered between October 2000 and November 2009 in Croatia ($n = 2$), Cyprus ($n = 8$), Greece ($n = 13$), Italy ($n = 73$), Portugal ($n = 3$) and Spain ($n = 21$) from both wild and farmed animals. Samples were collected during the routine diagnostic activity and epidemiological monitoring of the Istituto Zooprofilattico Sperimentale delle Venezie. The betanodaviruses characterized in this study, as well as their year of isolation, host source, country and GenBank accession number of each sequence, are reported in Supplementary Table S1.

2.2. Virus isolation and propagation in cell culture

Tissue samples (mainly brains) were homogenized at the mortar with sterile quartz sand and diluted 1:10 w/v with Eagle Minimum Essential Medium (E-MEM) containing 10% fetal calf serum and 2% antibiotic/antimycotic solution. Homogenized tissues were subsequently centrifuged 15 min at 4000*g*. After an overnight incubation at +4 °C, 100 μl of supernatant was inoculated into striped snakehead (SSN-1) cell monolayers, incubated at 25 °C for 10 days and checked regularly for cytopathic effect (CPE) (Frerichs et al., 1996). If no CPE appeared, samples were subjected to sub-cultivation into a new SSN-1 monolayer. Cell culture supernatants were recovered from positive samples showing typical intracytoplasmatic vacuoles, clarified by centrifugation at 3000*g* for 15 min at +4 °C and stored at −80 °C until use.

2.3. RNA extraction, RT-PCR and sequencing

Total RNA was extracted from 100 μl of cell culture supernatant using the NucleoSpin® RNA II (Macherey–Nagel GmbH & Co., Düren, Germany) according to the manufacturers instructions. Reverse transcription followed by PCR amplification was performed with the "Qiagen® OneStep RT-PCR kit" (Qiagen GmbH, Hilden, Germany) applying the following cycling conditions: 50 °C for 30 min, 95 °C for 15 min and 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 45 s elongation at 72 °C; the reaction was terminated with 10 min elongation at 72 °C. Primers used for the amplification of RNA1 and RNA2 partial sequences have been previously published by Toffolo et al. (2007) and Bovo et al. (2011). PCR products were analyzed for purity and size by electrophoresis in 2% agarose gel after staining with GelRedTM Nucleic Acid Gel Stain (Biotium, Hayward, CA). Amplicons were subsequently purified with ExoSAP-IT® (USB Corporation, Cleveland, OH) and sequenced in both directions using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The products of the sequencing reactions were cleaned-up using the Performa DTR Ultra 96-well kit (Edge BioSystems, Gaithersburg, MD) and analyzed on a 16-capillary ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.4. Phylogenetic analysis

Sequencing data were assembled and edited with the SeqScape® software v2.5 (Applied Biosystems) and the consensus sequences obtained were aligned and compared to reference nucleotide sequences available in GenBank using the MEGA 4 package (Tamura et al., 2007). For both the RNA1 and RNA2 partial segments, maximum likelihood (ML) trees were estimated using the PAUP* 4.0 package (Swofford, 2002) and employing the general time-reversible (GTR) + I + Γ_4 model of nucleotide substitution that was found to be the best-fit to the data in hand using MODELTEST (Posada and Crandall, 1998). Parameter values for the GTR substitution matrix, base composition, gamma distribution of among-site rate variation (with four rate categories, Γ_4), and proportion of invariant sites (I) were estimated directly from the data using MODELTEST and are available from the authors on request. A bootstrap resampling process (1000 replications) using the neighbor-joining (NJ) method and incorporating the ML substitution model defined above was used to assess the robustness of individual nodes on the phylogeny. Fixed amino acid changes along branches of the phylogeny were identified using the parsimony algorithm available in MacClade program (Maddison and Maddison, 1989).

2.5. Substitution rates and time-scale of evolutionary history

Rates of nucleotide substitution per site, per year and the time to Most Recent Common Ancestors (tMRCAs) of specific groups (i.e. RGNNV) were estimated using the BEAST program version 1.5.3 (Drummond and Rambaut, 2007) which employs a Bayesian Markov chain Monte Carlo (BMCMC) approach. All data sets were analyzed with the codon-based SRD06 nucleotide substitution model (Shapiro et al., 2006). For each analysis, the Bayesian skyline coa-

lescent tree prior was used as this is likely to represent the best description of the complex population dynamics of betanodavirus (Drummond et al., 2005). Two molecular clock models – strict (constant) and uncorrelated lognormal (UCLN) relaxed clock – were compared by analyzing values of the coefficient of variation (CoV) in Tracer (Drummond et al., 2006), in which CoV values >0 are evidence of non-clock-like evolutionary behavior. In all cases, uncertainty in the data is reflected in values of the 95% highest probability density (HPD) values for each parameter estimated, and in each case chain lengths were run for sufficient time to achieve coverage as assessed using the Tracer v1.5 program (Drummond and Rambaut, 2007).

2.6. Analysis of selection pressures

Gene and site-specific selection pressures for the RNA1 and RNA2 gene segments of all the betanodaviruses analyzed in this study were measured as the ratio of nonsynonymous (d_N) to synonymous (d_S) nucleotide substitutions per site (d_N/d_S). In all cases d_N/d_S ratios and the selection pressures at individual codons were estimated using the Single Likelihood Ancestor Counting (SLAC) and Fixed Effects Likelihood (FEL) methods available at the Datamonkey online version of the Hy-Phy package (Delport et al., 2010; Kosakovsky Pond and Frost, 2005). All analyses utilized the GTR model of nucleotide substitution and employed input neighbor-joining phylogenetic trees.

2.7. Phylogeny-trait association analysis

To determine the extent and pattern of geographical structure of betanodaviruses, we first grouped the sequences of viruses into nine geographic regions: North-eastern Italy, South-eastern Italy, South-western Italy, North-western Italy (as most of our samples came from Italy), Croatia, Cyprus, Greece, Portugal and Spain. Unfortunately, the exact place of location is unknown for most of the samples collected outside Italy. To assess the overall degree of geographical structure among the betanodaviruses sampled from Southern European countries, we used the BaTS 1.0 program (Parker et al., 2008) to estimate values of the association index (AI) and parsimony score (PS) statistics of phylogeny-trait association, with the trait (the geographical origin) as defined above. This method is able to account for phylogenetic uncertainty in the data by using the posterior distribution of trees obtained from the BEAST analysis described above. The BaTS program also allowed us to assess the level of clustering in individual locations using the monophyletic clade (MC) size statistic (Parker et al., 2008). In all cases, 1000 random permutations of tip locations were undertaken to create a null distribution for each statistic. An equivalent phylogeny-trait association analysis was performed to determine the extent of viral gene flow between feral and reared fish populations. In this case, the sequences of viruses were grouped into two categories, accordingly to the origin of the fish: farmed or wild.

3. Results

3.1. Viral isolation

We isolated betanodaviruses from 19 distinct fish species belonging to 7 orders and 12 families. Importantly, we identified viral strains in five fish species for the first time, namely triggerfish, *Balistapus* spp., thicklip grey mullet, *Chelon labrosus*, goldblotch grouper, *Epinephelus costae*, surmullet, *Mullus surmuletus* and sea trout, *Salmo trutta trutta*, and in three marine invertebrates (*Artemia salina*, *Opistobranchia* and *Ruditapes philippinarum*) (Table S1).

3.2. Phylogenetic analysis

The maximum likelihood phylogenetic trees inferred for the RNA1 and RNA2 genes of 120 viruses collected from six different European countries from 2000 to 2009 revealed that all betanodaviruses circulating in Southern Europe fell within two genotypes: RGNNV and SJNNV (Figs. 1 and 2). The topology of the RNA1 and RNA2 phylogenetic trees identified 11 (I–XI) and seven (A–G) well supported monophyletic genetic clusters (bootstrap values >70%), respectively (Figs. 1 and 2), although there is clearly an arbitrary element to this classification as some clusters can be further subdivided. Despite these limitations, these clusters greatly assist in the identification of inter-genotype reassortment events. Most notably, 23 of 24 viruses that belong to the SJNNV genotype (group G) in the RNA2 phylogenetic tree fell within the RGNNV genotype in the RNA1 tree. Interestingly, none of these viruses was isolated from wild fish. More specifically, 22 of 23 samples included in the RNA2-G group fell within cluster RNA1-VIII, while one reassortant strain circulating in Italy in 2005 grouped within cluster RNA1-IV. Strain 484.2.2009 (sampled from Spain), belonging to cluster XI and G in the RNA1 and RNA2 phylogenies, respectively, was the only one consistently genotyped as SJNNV in both trees. Similarly, intra-genotype reassortments were also observed within the RGNNV genotype. For instance, groups RNA2-B and RNA2-E identified in the RNA2 phylogeny, merged into a single cluster (IV) on the RNA1 tree (Table 1).

Viruses belonging to groups II, IV, V, VIII, IX in RNA1 and D, E, G, F in RNA2, appeared to have circulated extensively in Southern Europe, while strains included in clusters I, III, VI, VII, X for RNA1 and A–C for RNA2, were detectable only for a limited period of time (less than 3 years) (Table 1). Interestingly, a large number of amino acid substitutions were identified within the RGNNV genotype for both RdRp and CP gene segments (Figs. 1 and 2). In RNA1, the highest number of amino acid substitutions occurred along branches of group VIII (seven amino acid changes), which includes all the reassortant RGNNV/SJNNV viruses. In contrast, in RNA2 more amino acid changes occurred along branches of clusters including groups B–D (21 amino acid changes).

3.3. Geographical and ecological clustering

Although our phylogenetic analysis revealed some mixing of betanodavirus sequences among localities, such as groups IV (or E), VIII (or G) and XI (or D), which are clearly indicative of some viral gene flow among geographic locations, it was noticeable that most of the viruses sampled from individual countries tended to cluster together and are therefore strongly suggestive of an overall betanodavirus population subdivision. Indeed, 9 out of 11 (I, II, III, V, VI, VII, X and XI) and 4 out of 7 (A, B, C, F) genetic groups in the RNA1 and RNA2 phylogenies, respectively, were collected from one single country. To examine the extent and pattern of geographical structure of these data in a more quantitatively rigorous manner, we used a series of phylogeny-trait association tests in which each virus was assigned to a different geographic region (i.e. country or Italian region). This revealed a very strong geographic clustering of strains by area of origin ($P = 0$ for both AI and PS statistics in both gene segments). When the extent of phylogenetic clustering of individual regions was tested (using the MC statistic), population subdivision was significant for most of the localities, although samples from the North-West part of Italy showed evidence for more gene flow in both genes ($P = 1$).

Similarly, we investigated the extent of viral gene flow between reared and feral fish, assigning to each virus the trait "farmed" or "wild". This analysis similarly revealed a strong

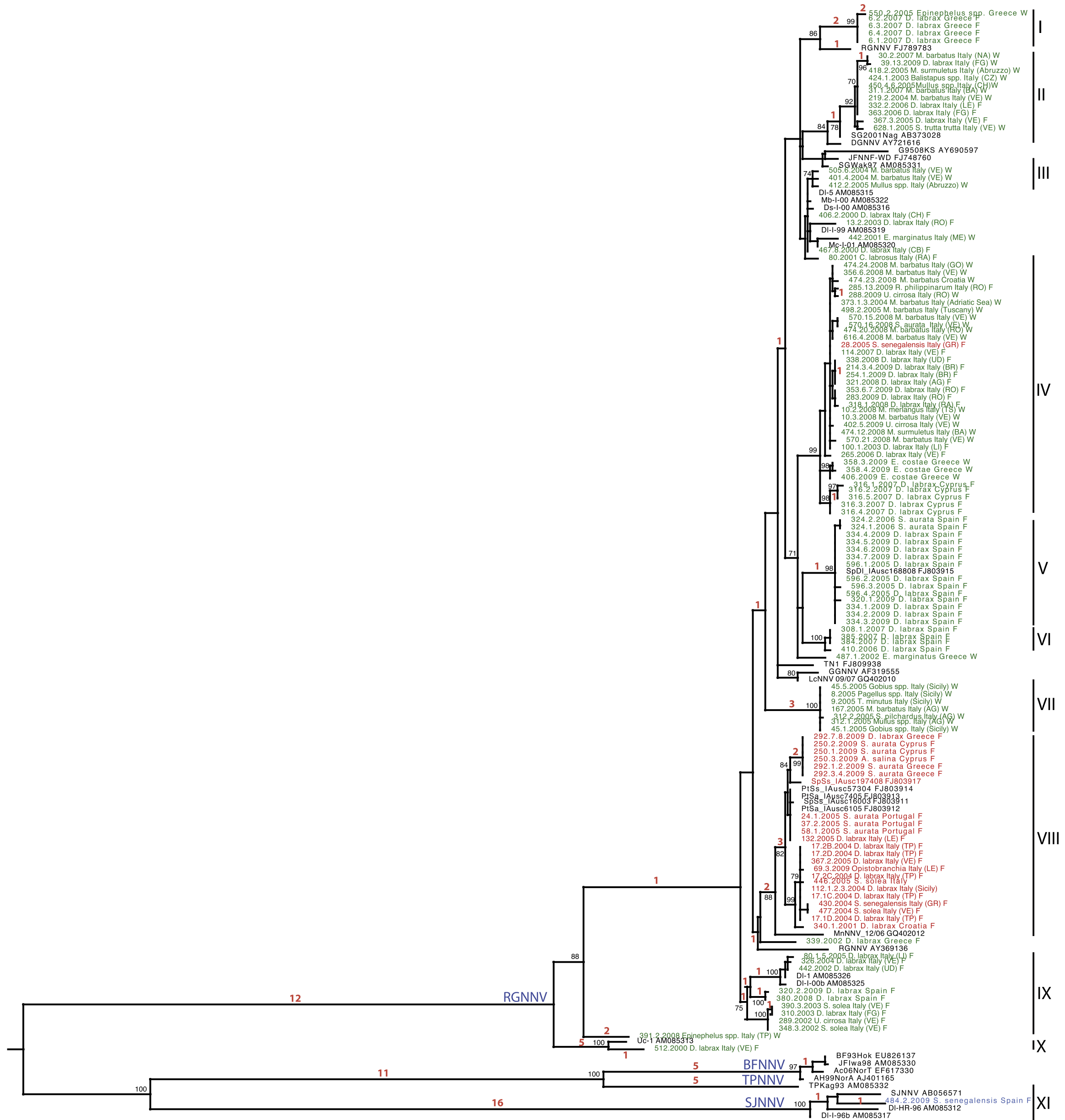

Fig. 1. ML phylogenetic tree of the RNA1 genetic segment of fish betanodaviruses collected between 2000 and 2009 from Croatia, Cyprus, Greece, Italy, Portugal and Spain, combined with sequences of representative genotypes. The sequence name includes: isolate identification number, host species, country of provenance and origin (F = farmed, W = wild). The numbers at branch points represent bootstrap values (black) and the number of nucleotide substitutions occurring along branches (red). Vertical bars designate genetic clusters (I–XI) and the genotype subdivision is shown at the main branches. Viral sequences analyzed in this study and typed as RGNNV, SJNNV and RGNNV/SJNNV are labeled in green, blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clustering of betanodaviruses accordingly to their farming status ($P = 0$ for AI and PS statistics in both gene segments). Indeed, for the RNA1 gene, the majority of genetic groups (8 out 11) were entirely collected from wild (groups III and VII) or farmed fish (groups V, VI, VIII, IX, X, XI), while for RNA2, 4 out 7 clusters (G, C, D, F) include only viruses isolated from farmed fish.

3.4. Evolutionary dynamics of betanodaviruses

Rates of nucleotide substitution were estimated for each gene segment of all the viruses analyzed in this study. For the RNA1 gene segment, the lower 95% HPDs of CoV value of the relaxed (uncorrelated lognormal) molecular clock was >0, so that a relaxed

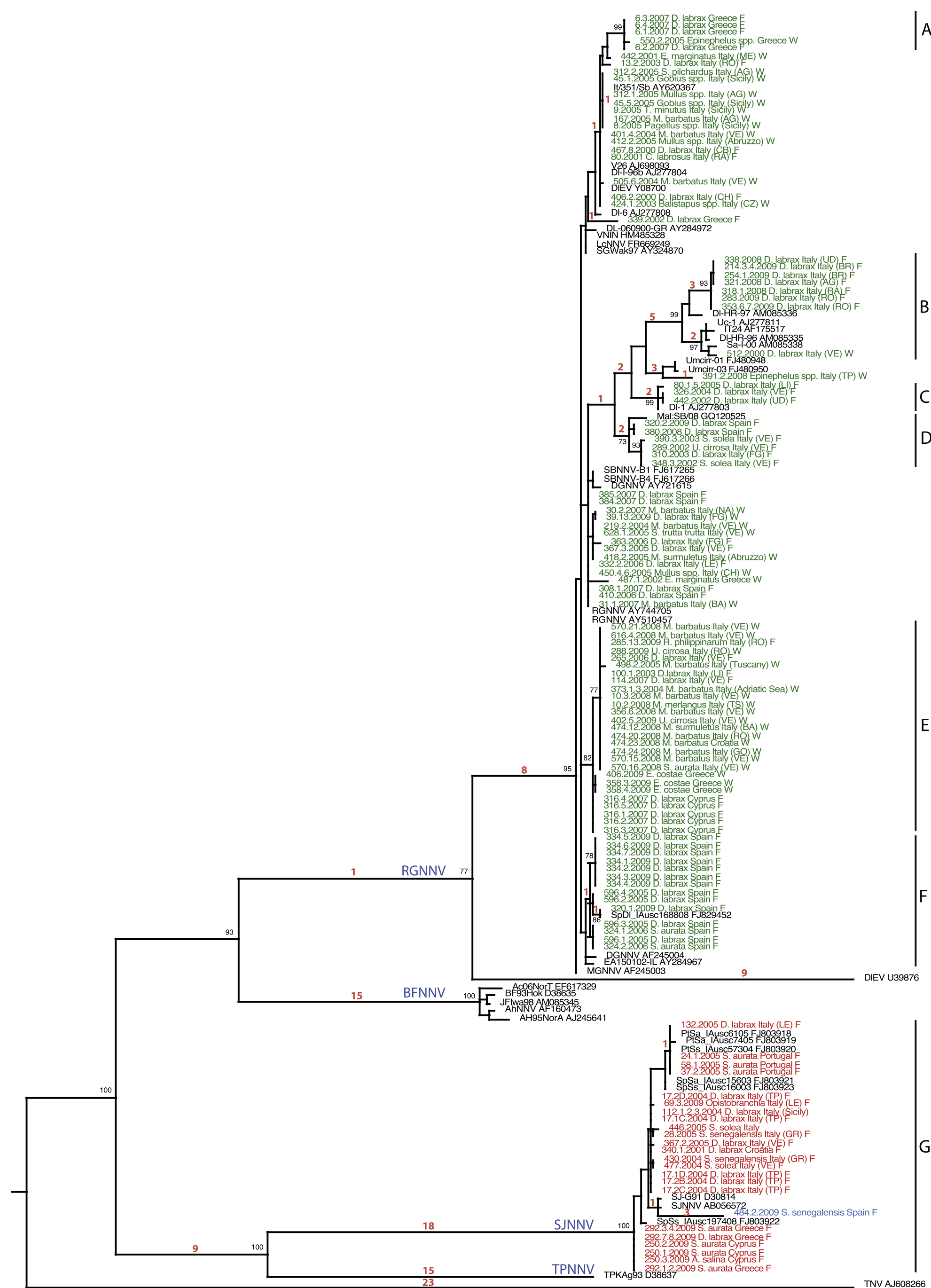

Fig. 2. ML phylogenetic tree of the RNA2 genetic segment of fish betanodaviruses collected between 2000 and 2009 from Croatia, Cyprus, Greece, Italy, Portugal and Spain, combined with sequences of representative genotypes. Sequences are named as in Fig. 1. The numbers at branch points represent bootstrap values (black) and the number of nucleotide substitutions occurring along branches (red). Vertical bars designate genetic clusters (A-G) and the genotype subdivision is shown at the main branches. Viral sequences analyzed in this study and typed as RGNNV, SJNNV and RGNNV/SJNNV are labeled in green, blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular clock model, which allows for the rate variation across lineages, was employed. In contrast, the RNA2 gene segment showed CoV value approximately of 0, and a strict molecular clock model was used to estimate evolutionary dynamics in this case.

Table 1
Clusters of betanodaviruses within the RNA1 and RNA2 topologies and their characteristics.

RNA1 cluster	RNA2 cluster	Country	Period	N. farmed fish	N. wild fish
I	A	Greece	2005–07	4	1
II	–	Italy	2003–09	3	8
III	–	Italy	2004–05	–	3
IV	B	Italy	2008–09	7	1[a]
	E	Croatia Cyprus Greece Italy	2003–09	9[b]	18
V	F	Spain	2005–09	14	0
VI	–	Spain	2006–07	4	0
VII	–	Italy	2005	0	7
VIII	G	Croatia Cyprus Greece Italy Portugal Spain	2001–09	23	0
XI		Spain	2009	1	0
IX	C	Italy	2002–05	3	0
	D	Italy Spain	2002–09	6	0
X	–	Italy	2000	1	0

[a] Sample 512.2000 is not included in the RNA1 cluster IV.
[b] Sample 28.2005 is included in the RNA2 cluster G instead of cluster E.

The mean rate of nucleotide substitution for the RNA1 gene was 4.89×10^{-4} substitution/site/year (95% HPD, 2.92×10^{-4} to 6.97×10^{-4}). Notably, a significantly lower rate of nucleotide substitution was found for the RNA2 gene, at 2.42×10^{-4} substitution/site/year (95% HPD, 1.07×10^{-4} to 3.99×10^{-4}). Under these rates, the mean tMRCA calculated for the RNA2 of the larger RGNNV genotype ranged from 79 to 328 years ago (95% HPD values, mean of 188 years), which overlap with the tMRCA obtained for the RNA1 gene of the same genotype, which ranged from 63 to 211 years ago (95% HPD values, mean of 128 years).

3.5. Selection pressures on the RNA1 and RNA2 genes and unique amino acid signatures

Most codons in the RNA1 and RNA2 segments were subject to relatively strong purifying selection; mean d_N/d_S ratios were 0.093 for RNA1 and 0.178 for RNA2. However, we identified three codons in the coat protein gene and two in the viral polymerase gene that may be subject to positive selection (p-value < 0.1). Specifically, all three putatively positively selected residues in the RNA2 segment – positions 238, 291 and 292 – are located at the C-terminal protruding portion of the coat protein (from amino acid 238 to 340) (Iwamoto et al., 2004). Site 238 fell within the 232–255 amino acidic region (nt 695–765) previously described by Ito et al. (2008) as one of the major putative host-specificity determinants. Indeed, we observed a high number of substitutions within the C-terminal region (aa 238–340) in several viruses belonging to the RGNNV genotype. Specifically, genetic groups B–D showed 13 mutations in this region, and which might also reflect some localized selection (Fig. 2). Similarly, of the 28 amino acid mutations that distinguish the SJNNV and RGNNV genotypes (Fig. 2), 22 fell within the C-terminal protruding region (aa 238–340). The RNA1 gene contained two putatively positively selected sites, at positions 41 and 48. As both positions fell within a region (amino acids 1–445) that plays an important role in determining temperature sensitivity (Hata et al., 2010), it possible that they also effect this aspect of phenotype. In the same region, 34 amino acid substitutions are observable within the RGNNV genotype (Fig. 1).

4. Discussion

We collected a total of 120 betanodavirus isolates from 2000 to 2009 in Southern Europe and undertook a variety of analyses of their molecular evolution and epidemiology. To our knowledge, this is the largest study of both genomic segments undertaken to date. Viral strains were isolated from a multiplicity of fish samples belonging to nine distinct orders; *Clupeiformes*, *Gadiformes*, *Mugilformes*, *Perciformes*, *Pleuronectiformes*, *Salmoniformes* and *Tetraodontiformes*. Of note, betanodaviruses have been identified for the first time in this study in six different species, extending the list of susceptible fish species to VER infection. Unfortunately, clinical information was available only for a limited number of samples (n = 32) (data not shown), therefore it was not possible to associate the virus genetic features with virulence. Betanodavirus was also isolated from three marine invertebrates, as previously reported by Gomez et al. (2008b). The role of such contaminated organisms in the spread of the virus remains uncertain, and it is possible that they constitute an infection source for wild and reared fish populations.

Our phylogenetic analysis of the RNA1 and RNA2 segments revealed a preponderance of the RGNNV (n = 96) genotype over the SJNNV (n = 1) genotype in Southern Europe. Interestingly, 23/120 samples were RGNNV/SJNNV reassortant strains. Such chimeric viruses were previously described by Olveira et al. (2009) in gilthead seabream and Senegalese sole. The present study confirms the susceptibility of these species to the infection with the reassortant RGNNV/SJNNV, in addition to sea bass, common sole, and two marine invertebrates which were described here for the first time. The relative high frequency of reassortant strains highlights the importance of conducting phylogenetic analyses of both genomic

segments. Indeed, the phylogenetic analysis solely based on the coat protein gene may be misleading as demonstrated by Olveira et al. (2009), who revealed seven isolates previously classified as SJNNV from an RNA2 analysis by Cutrín et al. (2007), to in fact be reassortants. Finally, the combined characterization of RNA1 and RNA2 definitively confirms the presence of the SJNNV genotype in Southern Europe.

Importantly, the phylogeny-based analyses of geographical association revealed that viruses sampled from individual countries tended to cluster together, indicative of major population (geographic) subdivision among betanodaviruses, although some limited examples of viral gene flow were also observed. Indeed, few genetic clusters included viruses isolated from single geographic areas, suggesting the existence of epidemiological and commercial connections among different countries. Similarly, our analysis revealed a strong clustering of betanodaviruses accordingly to their farming status (wild or farmed). In only a few cases the viruses isolated from feral and farmed animals were found to be highly similar, indicative of migration among these fish populations. Unfortunately, our data does not shed light on the means by which the virus was introduced into specific geographic regions, as neither of the two main means of spread – through wild fish or trade of reared animals – can be definitely excluded on these data, particularly as there is strong clustering by both characteristics. This is clearly an issue that requires further study.

Although the mean d_N/d_S ratio as well as the number of positively selected sites is greater for the viral capsid than the polymerase, suggesting that the former protein is subject to stronger positive selection pressure, it was striking that the viral polymerase gene evolved significantly more rapidly than the coat protein gene. One possible explanation for this difference in evolutionary rates is that there has been adaptation to local temperature conditions which is known to modulate viral RNA replication and which is under control of RNA1; indeed, we observed positively selected sites within a genomic region of the polymerase gene previously shown to be important in determining the temperature sensitivity of betanodaviruses (Hata et al., 2010).

Three positively selected sites were identified within the major host specificity determinant of the protruding region of the coat protein gene (Ito et al., 2008). Interestingly, we observed no species-specific mutations in this RNA2 region, and there appeared to be no correlation between viral strain and specificity in host tropism, since the viruses with the same genetic characteristics are capable to infect species belonging to different taxonomic orders. In addition, a large number of nucleotide mutations were documented in clades B and C of the RNA2 tree, which include viruses isolated only from farmed sea bass. This suggests that mutations are not necessarily fixed during host shifts. Indeed, the genetic clusters associated with high levels of genetic diversity – either nucleotide mutations or genetic reassortment (namely B, C, D and G) – consist of betanodavirus strains isolated only from farmed animals, mainly sea bass. This scenario highlights that environmental and housing conditions peculiar to fish farms (such as stress factors, population density per m^3, introduction of new fish batches) might play an important role in viral evolution and perhaps facilitating frequent genetic reassortment.

5. Conclusions

We provide a more complete understanding of the distribution of different betanodavirus genotypes in Southern Europe and hence of the molecular epidemiology of VER, highlighting the frequent occurrence of genetic reassortment. Our findings suggest that molecular investigations such as this are essential to the development of adequate surveillance strategies for this disease and provide significant information on the epidemiology of this virus. The present study also makes available valuable data on the evolutionary dynamics of betanodaviruses, serving as a useful substrate for further studies using reverse genetics based systems to study the effect of specific mutations and genetic reassortment on key aspects of viral phenotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.10.007.

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Chapter 1

General introduction and aims

1. Background

Viral nervous necrosis (VNN) (synonymous viral encephalopathy and retinopathy – VER) is a severe neuropathological disease caused by small RNA viruses belonging to the family *Nodaviridae*, genus *Betanodavirus*. After their first detection in the early nineties, betanodaviruses have rapidly spread worldwide representing a threat both for marine aquaculture and for wild fish populations. VNN has been recently included among the most significant viral pathogens of finfish, due to its severity, the broad host range and the lack of prophylactic measures (Rigos and Katharios, 2009; Walker and Winton, 2010; Terlizzi et al., 2009). Indeed, despite VNN was removed from the list of the OIE notifiable diseases in 2006, the VNN chapter as well as the two OIE reference laboratories have been maintained considering the impact the disease may have on marine aquaculture.

2. VNN aetiology

The causative agent of VNN is a small (25-30 nm diameter), spherical and non-enveloped viral particle, with a bi-segmented RNA genome (King et al., 2014). The family name *Nodaviridae* derives from the Japanese village Nodamura, were the prototype virus (Nodamura virus, NoV) was isolated from mosquitos *Culex tritaeniorhynchus* (Scherer and Hurlbut, 1967). In 1992, Mori and collaborators identified a different nodavirus from fish larvae of striped jack (*Pseaudocaranx dentex*), which explains the name of the betanodavirus type species striped jack nervous necrosis virus (SJNNV). Subsequent molecular studies discriminated these two viruses into two different genera: the *Alphanodavirus* and the *Betanodavirus,* infecting insects and fish, respectively (Nishizawa et al. 1995). A third genus, the *Gammanodavirus*, has been recently added to the *Nodaviridae* family, comprising viruses detected in prawns (*Macrobranchium rosenbergii*) (NaveenKumar et al., 2013).

2.1. Betanodavirus genome

The genome of betanodavirus is formed by two single-stranded positive-sense RNA molecules with a Cap structure at the 5'ends. The RNA1 segment (3.1 Kb) encodes for the RNA-dependant RNA-polymerase (RdRp) also called protein A, and the RNA2 segment (1.4 Kb) encodes for the structural capsid protein (Mori et al., 1992; Sommerset and Nerland, 2004). A further subgenomic transcript of 0.4 Kb, called RNA3, originates from

the RNA1 molecule during active viral replication and encodes for the B2 protein, which antagonizes host cell RNA interference mechanisms (Fenner et al., 2006a; Iwamoto et al., 2005; Sommerset and Nerland 2004). The transcription of the RNA1 segment seems to occur during the early stages of viral cycle, whereas the expression of the capsid protein, and thus the increase in the number of infective virions, takes place at a later stage (Lopez-Jimena et al., 2011). Sommerset and Nerland (2004) have demonstrated that RNA3 synthesis during betanodavirus replication in cell culture is much more abundant when compared to the RNA1 transcription at an early point of infection. In agreement with these observations, Mézeth et al. (2009) showed that the B2 non-structural protein is detected only during the inner phase of the infection, both *in vitro* and *in vivo* while the betanodavirus capsid protein is present also in chronically infected fish that have survived the acute phase of the disease.

2.2. Genetic and serological classification of betanodaviruses

According to the phylogenetic analysis of the T4 variable region within the RNA2 segment, betanodaviruses can be classified into four different species: the striped jack nervous necrosis virus (SJNNV), the tiger puffer nervous necrosis virus (TPNNV), the barfin flounder nervous necrosis virus (BFNNV) and the red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). Furthermore, a single betanodavirus from turbot (*Scophthalmus maximus*) has been suggested as a fifth species (Johansen et al., 2004). The genetic variability observed among fish nodaviruses is further increased by genetic drift and genetic shift phenomena. As a matter of fact, if on one hand we may observe the fixation of mutations which characterize specific viral groups, on the other, genetic inter or intra-genotype reassortment events may generate major changes, to the point of creating viral strains with new genetic and phenotypic features (He et al., 2015; Panzarin et al., 2012). Several authors (Olveira et al., 2009; Panzarin et al., 2012; Toffolo et al., 2007) have described the existence of two reassortant viruses deriving from the genetic mixing of the RGNNV and the SJNNV genotypes. The RGNNV/SJNNV reassortant harbours the polymerase gene of the RGNNV and the coat protein gene of the SJNNV, while the reassortant SJNNV/RGNNV possesses the RNA1 segment of the SJNNV and the RNA2 gene of the RGNNV. These reassortant strains may have been generated by the coexistence of two different viral species in the same host (Lopez-Jimena

et al. 2010; Sakamoto et al., 2008). Antigenically, betanodaviruses share a certain number of epitopes but are only partially cross-reactive. In detail, fish nodaviruses can be grouped into three distinct serotypes: serotype A, which includes viruses belonging to the SJNNV genotype, serotype B, which comprises the TPNNV genotype, and serotype C consisting of viruses belonging to the RGNNV and BFNNV genotypes (Mori et al., 2003).

2.3. Structural characterization of betanodavirus

The crystal structure of virus-like particles (VLPs) of malabaricus grouper nervous necrosis virus (MGNNV) was determined by electron cryomicroscopy (cryoEM) and three-dimensional reconstruction (Tang et al., 2002). The proposed model suggests that fish nodaviruses possess a capsid subunit domain organization different from that of insect nodaviruses, where the coat protein has two domains: the β-sandwich domain and the trimeric protrusion domain. More recently, Chen et al. (2015) resolved the crystal structure of a grouper nervous necrosis virus-like particle (GNNV-LP). The topological structure of the GNNV coat protein consist of an N-terminal arm (aa 34-51), and a shell domain (S-domain) (aa 52-213) connected through a linker region (aa 214-220) to a protrusion trimeric domain (P-domain) (aa 221-338). This latter contains a motif for calcium binding, and plays an important role in the trimerization of the GNNV CPs during the initial capsid assembly process. Furthermore, Chen et al. (2015) reported that the hypervariable regions of the P-domain correspond to coat protein portions involved in the receptor binding and the host tropism of betanodavirus. Indeed, through the generation of reverse genetics viruses, Ito et al. (2008) showed that the nucleotide sequences 694-1054 and 695-1061 (and/or their encoded amino acids) of the RGNNV and the SJNNV, respectively, are required to recognize the homologous host species. Furthermore, pairwise surface probability plots for the SJNNV and the RGNNV CPs suggest a structural difference of the capsids of these two genotypes.

3. Geographical distribution and host tropism of betanodavirus

Since its first description in a European seabass hatchery in French Martinique (Bellance and Gallet de Saint-Aurin, 1988) in the late ‘80s, VNN appeared almost simultaneously also in Asia (Mori et al., 1992), Australia (Glazebrook et al., 1990) and southern Europe (Breuil et al., 2001,). Later on, the disease further spread in North America and northern

Europe (Barke et al., 2002; Curtis et al., 2001; Grotmol et al., 2000, Starkey et al., 2001). Nowadays, VNN is present all over the world, having become endemic in several areas and affecting almost all marine farmed species and a number of wild species as well.
The RGNNV is the most common genotype described in clinical outbreaks in the Mediterranean region, in south-eastern Asia and Australia (Binesh et al., 2013; Cherif et al., 2009; Gomez et al., 2008a and b; Olveira et al., 2009; Panzarin et al., 2012; Ransangan et al., 2012; Sakamoto et al., 2008; Skliris et al., 2001; Ucko et al., 2004), showing also the broadest host range and the widest temperature tolerance. The SJNNV genotype has been detected only in Japan and Spain (García-Rosado et al., 2007; Sakamoto et al., 2008; Skliris et al., 2001). Noteworthy, VNN outbreaks resulting from reassortant strains have been described only in the Mediterranean Sea (He et al., 2015); in particular, RGNNV/SJNNV epidemics are quite common in the Iberian Peninsula and in Italy (Olveira et al., 2009; Panzarin et al., 2012). The BFNNV circulates in cold waters of the Northern Atlantic, the North Sea and Japan (Grotmol et al., 1997; Nguyen et al., 1994; Nylund et al., 2008; Sakamoto et al., 2008), while the TPNNV virus occurred only once in Japan (Nishizawa et al., 1994). The geographic distribution of different betanodavirus genotypes clearly reflects their water temperature preferences, as a result of viral adaptation to different environmental conditions. Interestingly, genetically different betanodaviruses behave differently in response to diverse incubation temperatures *in vitro*. Indeed, BFNNV and TPNNV are considered psychrophilic, with optimal culture temperature ranging from 15° to 20° C. On the other hand, the SJNNV strain shows its best replication fitness in cell culture at 25°C. The RGNNV species displays the widest tolerance, being capable to replicate between 15°C and 35°C, with an optimum growth temperature comprised between 25 and 30°C (Ciulli et al., 2006a; Hata et al., 2010; Iwamoto et al., 2000). In a recent study, through the engineering of reverse genetics viruses, Hata and collaborators (2010) showed that the incubation temperature affects betanodavirus replication, and that the RNA1 genetic segment and its encoded protein play a major role in controlling the temperature sensitivity of fish nodaviruses. In particular, the amino acid residues 1-445 of the viral replicase appear to be sufficient to control temperature tolerance of betanodavirus, putatively affecting the transfer of protein A to mitochondrial membranes, where the replication occurs (Hata et al., 2010, Guo et al., 2004, Mézeth et al., 2007).

VNN is mostly a disease of the marine environment; the list of susceptible species has expanded in the past years, and also species historically considered resistant to the disease have been included, as in the case of sea bream (Beraldo et al., 2011; Toffan, personal communications). To date, betanodavirus has been detected in 165 species belonging to 81 families and 24 orders. Among the VNN susceptible species, the most frequently reported belong to the families *Carangidae, Percichthydae, Serranidae, Sciaenidae, Pleuronectidae, Mugilidae, Sebastidae,Gadidae.* Indeed, the most commonly and severely affected species are sea bass (*Dicentrarchus labrax* and *Lates calcarifer*), groupers (*Ephinephelus* spp.), flatfish (*Solea* spp.*, Scophthalmus maximus, Paralichthys olivaceus*), striped jack (*Pseudocaranx dentex, Trachinotus spp.*) and drums (*Umbrina cirrosa, Argyrosomus regius, Sciaenops ocellatus, Atractoscion nobilis*). Notably, an increasing number of outbreaks in freshwater species has also been reported. Indeed, several studies have highlighted the capacity of VNN to cause disease and infect a great number of fish species independently from water salinity, which does not seem to affect virus infectivity (Furushawa et al., 2007; Korsnes et al., 2005; Maeno et al., 2007; Pascoli et al., 2016). The detection of betanodavirus in wild asymptomatic fish is also extensively documented (Ciulli et al., 2006b; Giacopello et al., 2013; Gomez et al., 2004 and 2008b; Nylund et al., 2008; Panzarin et al., 2012; Sakamoto et al., 2008) which explains the wide diffusion of this viral agent in different continents. The increasing number of VNN outbreaks in wild marine fish, especially groupers, associated to the presence of typical nervous signs, severe mortality and to the detection of the aetiological agent, is a matter of great concern (Gomez et al., 2009; Kara et al., 2014).

4. The disease

4.1. Clinical signs

VNN clinical signs include skin color variations, anorexia, lethargy, hyperinflation of the swim bladder, abnormal swimming behavior and nervous symptoms caused by the lesions in the brain and retina. (Bovo 1999; Breuil et al., 2001; Hellberg et al., 2010). In larvae/juveniles, the onset of the disease can be hyper-acute, with a sharp increase in mortality being the only apparent clinical sign. In elder animals, the onset of the disease and the cumulative mortality can be lower, but in this case a secondary effect of the infection reduces fish growth and increases the unevenness in weight/size .

In European sea bass (*Dicentrarchus. labrax*), typical clinical signs are loss of appetite, skin darkening, swirling, circular movements alternated to long periods of lethargy, abnormal bathymetry and anomalous vertical positions in the water column. Fish appear blind and can display hyper-excitability when disturbed (Peducasse et al., 1999; Athanassopoulou et al., 2003). Traumatic lesions to the jaw, head, eyes and nose are the natural consequence of the impaired swimming capacity and blindness (Le Breton et al., 1997; Maltese and Bovo, 2007). In larvae and juveniles, congestion of the brain or of the whole head can be observed. Mortality can vary between 10% and 100%, depending on age and water temperature (Cherif et al., 2009; Munday et al.; 2002). Notably, higher the temperature, quicker is the disease progression and severity (Bovo et al., 1999; Breuil et al., 2001; Cherif et al., 2009; Le Breton et al., 1997).
Groupers, members of genus *Epinephelus*, are amongst the most susceptible fish species to VNN. In these animals the disease has been observed in all age groups, both in farmed and wild animals. In farmed *E. akaara* and *E. fuscoguttatus* larvae, the first signs of disease were loss of equilibrium, swimming in a corkscrew fashion and lethargy associated with abnormal response to stimulation. Fish sank to the bottom and then floated back to the surface. A few specimens were darker than usual and mortality was 95% (Chi et al., 1997). Severe hyperinflation of the swim bladder and corneal opacity is generally the most relevant clinical signs observed in both farmed and wild fish (Gomez et al., 2009; Kara et al., 2014; Pirarat et al., 2009; Sohn et al., 1998), although spinal deformities and exophthalmos were reported, as well (Sohn et al., 1998).
Less evident symptoms usually appear in flatfish: they remain on the tank bottom and bend their body with head and tail raised, or laying upside down. They may tremble or drop to the bottom of the tank with a typical "falling leaf" movement (Maltese and Bovo 2007). Skin discoloration has been noticed in Atlantic halibut *Hippoglossus hippoglossus* (Grotmol et al., 1997). It has been reported that during a natural outbreak, *Solea solea* appeared either darker or paler than usual, at times anorexic, with cumulative mortality equal to 100% (Starkey et al., 2000). In addition, in *Solea senegalensis* the presence of skin ulcers were also observed (Olveira et al., 2008). This latter species is extremely susceptible to the reassortant strain RGNNV/SJNNV, with 100% mortality at a water temperature of 22°C. Mortality can be dramatically reduced down to 8% when housing temperature is lower (16°C). Notably, the rise of water temperature caused a significant increase of the

viral load in fish challenged at 16°C, with a consequent escalation of mortality cases. This means that VNN can cause a persistent infection in Senegalese sole at low temperatures, and the virus can easily be reactivated by an increase of water temperature even a long time after the infection (Souto et al., 2015a and b).

In cold water species, such as Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*), fish can show clinical signs at 6°-15°C, displaying lethargy, anorexia, darkening of the skin and nervous abnormalities (Grotmol et al., 1997; Patel et al., 2007). Swim bladder inflammation and corneal opacity were also reported (Hellberg et al., 2010).

Fish belonging to the *Sparidae* family are historically considered as being resistant to clinical disease but not to the infection and this is why they may act as healthy carries. Gilthead sea bream (*Sparus aurata*) has often been reared in cohabitation with betanodavirus-infected European sea bass within the same farm, but no mortality nor clinical signs have been reported so far in this species (Aranguren et al., 2002; Beraldo et al., 2011; Castric et al., 2001;Ucko et al., 2004). Nevertheless, sea bream appeared to be susceptible to experimental infections by intramuscular injection with RGNNV strain, displaying mortality in juveniles or acting as an asymptomatic carrier for sea bass (Aranguren et al., 2002; Castric et al., 2001). Recently, an increasing number of VNN outbreaks in sea bream larvae has been reported (Beraldo et al. 2011; Toffan, personal communications). Interestingly, in all the disease outbreaks reported so far, viral strains isolated in sea bream have been characterized as being RGNNV/SJNNV. This suggests that a special adaptation of this type of reassortant to sea bream may be possible, as previously reported also for the Senegalese sole (Toffan, personal communications; Souto et al. 2015a).

4.2. Gross and microscopic lesions

VNN infected fish externally show few or no pathological lesions. Congestion, abrasion and sometimes necrosis of the nose, jaws and head have been observed in symptomatic *D. labrax* (Bovo et al., 1999; Le Breton et al., 1997). This could be due either to the congestion of the nervous tissue caused by the viral infection, or to the traumatic lesions caused by the abnormal swimming (Le Breton et al., 1997; Maltese and Bovo, 2007). Corneal opacity has been reported in groupers and European sea bass (Athanassopoulou et

al., 2003,Gomez et al., 2009, Kara et al., 2014; Pirarat et al., 2009). Skin erosions on body and fins caused by the impaired swimming ability are often commonly reported in these species (Maltese and Bovo, 2007; Kara et al., 2014). At necropsy the hyper-inflation of the swim bladder, signaled in almost all susceptible species, is generally the most prominent lesion together with the congestion of the central nervous system (CNS) and meninges.
Histological analyses on CNS reveal the presence of encephalitis characterized by multiple intracytoplasmatic vacuolation. Several empty areas with a diameter of 5-10 μm are present in the grey matter of the olfactory bulb, telencephalon, diencephalon, mesencephalon, cerebellum, medulla oblongata, spinal cord, retina and optic nerve. (Bovo et al., 1999; Le Breton et al., 1997; Mladineo 2003, Mori et al., 1992, Lopez-Jimena et al., 2010; Ucko et al., 2004). In the dendritic cell of the spinal cord, particularly in the cranial part, vacuolar lesions confirmed also by IHC were observed (Grotmol et al., 1997). Although the severity of the vacuolization depends on the fish species, age and stage of infection, such a lesion is a consistent finding (almost pathognomonic) in VNN cases. Pyknosis, karyorrhexis, neuronal degeneration and inflammatory infiltration has been observed in all the nervous tissues of the infected fish. Congestion of the blood vessels in the encephalic parenchyma and meninges is frequent and may evolve in mild or extensive hemorrhages in the brain (Korsnes et al., 2005; Mladineo et al., 2003; Pirarat et al., 2009).
The presence of viral antigens can be highlighted by immunohistochemistry (IHC) or *in situ* hybridization (ISH) in the molecular layer of CNS, optic tectum and cerebellum mainly surrounding the vacuoles but also when these are lacking (Lopez-Jimena et al., 2010; Mladineo et al., 2003, Pirarat et al., 2009). In some instances, viral particles have also been detected by IHC/ISH or PCR in non-nervous tissues, such as gills, fins, heart, anterior and posterior intestine, stomach, spleen, liver, kidney and gonads of several fish species (Grotmol et al., 1997; Grove et al., 2006; Johansen et al., 2003; Korsnes et al., 2005; Mazelet et al., 2011; Mladineo et al., 2003; Nguyen et al., 1997).

4.3. Transmission routes

Transmission of the disease occurs mainly horizontally through the direct contact with infected fish, infected water or contaminated equipment. As a matter of fact, the disease can easily be experimentally reproduced by bath (Castric et al., 2001; Johansen et al., 2003; Kai et al., 2008; Nguyen et al., 1996; Peducasse et al., 1999; Souto et al., 2015a).

Generally speaking, the younger is the fish, the more susceptible to the infection. Evidence of oral transmission through infected fish or contaminated feed batches has been also suggested (Peducasse et al., 1999; Skliris and Richards, 1999) Notably, the virus has also been detected in several marine invertebrates (Fichi et al., 2015; Gomez et al., 2006 and 2008b, Panzarin et al., 2012). The viral agent presents a very high resistance to chemical and physical agents; therefore, it can easily contaminate marine water, invertebrates and microorganisms but also nets, pens, tanks and other equipment (Maltese and Bovo, 2007).
Wild and farmed fish which have survived the disease may be recognized as putative carriers of virus into a free farm (Baek et al., 2007; Castric et al., 2001; Cutrin et al., 2007; Johansen et al., 2003; Skliris and Richards, 1999). It has been demonstrated that the disease and the viral shedding in persistently infected fish may be re-activated several times after the first onset due to stress factors or water temperature variations (Johansen et al., 2004; Souto et al., 2015b).
Vertical trasmission has also been described (Mazelet et al., 2011; Watanabe et al., 2000). The virus was detected in gonads and seminal fluids, although it has not been ascertained whether it was a real intra-ovo transmission or just an external contamination infecting the new-born larvae (Mushiake et al., 1992; Nguyen et al., 1997). However, the detection of betanodavirus through immunostaining in reproductive tissues such as the ovary, strongly supports the vertical transmission of the disease (Azad et al., 2006; Nguyen et al., 1997).

4.4. Pathogenesis

Several studies have tried to explain the pathogenesis of the VNN, although important viral mechanisms essential for the development of the disease still remain unclear. At a cellular level, there is only one paper reporting the identification of the GHSC70 heat shock protein commonly expressed by GF-1 cell line as a good betanodavirus receptor candidate (Chang and Chi 2015). Nevertheless, there may be other membrane proteins acting as viral receptor, of which we are presently unaware.
At a tissue level, the neurotropism of betanodaviruses is irrefutable, but how the virus reaches the nervous tissues is still a debated matter of discussion. It is believed that several tissues may act as portal of entry for betanodavirus, such as the nasal cavity, the intestinal epithelium and the skin. Tanaka et al. (2004) observed intranasally infected sevenband groupers and assumed that the virus had first penetrated into the nasal epithelium, then

reached the olfactory nerve and bulb, and finally invaded the olfactory lobe, which appeared to be the main site of viral replication. The nasal cavity as possible route of entry was assumed also in naturally infected European sea bass larvae (Mladineo et al., 2003) and in spotted coral groupers (Pirarat et al., 2009). In larvae and juveniles, possible entry portals may have been the epithelial cells of the skin or the intestinal epithelium, as observed in experimentally infected Atlantic halibut (Grove et al., 2003). Finally, the positive IHC reaction of the skin and the lateral line were also described (Grotmol et al., 1997; Nguyen et al., 1997, Peducasse et al., 1999), which suggest that skin epithelial cells might serve as portal for viral entry.

Once in the host, betanodavirus multiplies and spreads to the target organs through a viraemic phase, as extensively demonstrated by the viral detection in the blood (Korsnes et al., 2009, Lu et al., 2008; Lopez-Jimena et al., 2010; Olveira et al., 2008). Recurrent endocardial lesions, probably due to viraemia, have been reported in Atlantic halibut (Grotmol et al., 1997). Another hypothesis suggests that betanodaviruses may reach the central nervous system by axonal transport through the cranial nerves (Grotmol et al., 1999; Husgaro et al., 2001; Tanaka et al., 2004). The virus can then actively replicate into its target nervous organs, where the occurrence of vacuolization is generally associated with the appearance of clinical signs (Azad et al., 2006).

Betanodavirus infection strongly stimulates the innate immunity (Chen et al., 2014; Lu et al., 2008; Scapigliati et al., 2010). Following the first aspecific immune response, the development of a certain degree of circulating anti-betanodavirus antibodies was observed in different fish species. *In vitro*, induced antibodies appeared able to neutralize the viral activity, but it is still not clear whether they can overcome the hemato-encephalic barrier *in vivo* to protect the CNS from damage and clear the infection.

5. Diagnosis of the infection

The gold standard for the diagnosis of VNN is the isolation of the aetiological agent in susceptible continuous cell lines. The most commonly used are the SSN-1 cells, derived from the Snake head fish (*Ophicephalus striatus*) (Frerichs et al., 1996) and their derived clone E-11 (Iwamoto et al., 2000). The high susceptibility of these two cell lines has been attributed to the persistent infection with the Snakehead retrovirus (SnRV) (Lee et al., 2002; Nishizawa et al., 2008). Other continuous fish cell lines have been developed and

used successfully, but their diagnostic applications are presently unknown. The temperature of incubation is decisive for a successful viral isolation, which depends on the betanodavirus genotype (Ciulli et al., 2006a; Hata et al., 2007; Iwamoto et al., 2000). Cytopathic effect (CPE) is evident by the dark, granular and contracted cells which tend to detach from the surface of the flask. The occurrence of vacuoles inside the cytoplasm of infected cellsis observed. CPE further evolves into extended necrotic foci, resulting in the complete disruption of the cell monolayer.

Serological methods for the detection of betanodavirus include serum neutralization (SN) with monoclonal or polyclonal antibodies (Mori et al., 2003), indirect fluorescent antibody test (IFAT) (Castric et al., 2001; Mori et al., 2003; Peducasse et al., 1999) and enzyme-linked immunosorbent assay (ELISA) (Fenner et al., 2006b). The immunoenzimatic assay can be used directly on the central nervous system of the infected fish (Breuil et al., 2001; Nuñez-Ortiz et al., 2015) as well as IFAT (Curtis et al., 2001; Johansen et al., 2003; Nguyen et al., 1996; Totland et al., 1999).

The prompt diagnosis of VNN is now possible thanks to the development of molecular tests, i.e. the reverse transcriptase polymerase chain reaction (RT-PCR) and the more sensitive and rapid real-time RT-PCR. Conventional PCR using the F2-R3 primer set targeting the T4 variable region of the RNA2 segment has been widely applied for diagnostic purposes (Nishizawa et al., 1994). However, on some occasions sequence mismatches have caused the failure of these primers (Anonymous, 2013; Thiéry et al. 1999). A number of other PCR-based protocols have been developed to implement the sensitivity and the specificity of betanodavirus diagnostics, some of which have already been validated (Dalla Valle et al., 2005; Hick et al., 2010; Hodneland et al., 2011; Panzarin et al., 2010). Viral genome amplification might be followed by sequencing analysis which, besides being useful for diagnosis confirmation and virus identification, is also crucial for epidemiological studies. Notably, the phylogenetic analysis based on both genetic segments is advisable if we want to identify possible reassortment events between different betanodavirus genotypes or within the same genotype (Olveira et al., 2008; Panzarin et al., 2012; Toffolo et al., 2007).

Seroconversion in experimentally immunized/infected fish has been reported by several authors. ELISA is the most popular test for the analysis of unknown fish sera and guarantees a high sensitivity in different species (Breuil et al., 2000; Mushiake et al., 1992;

Watanabe et al., 2000; Scapigliati et al., 2010). SN is also suitable to evaluate the humoral response of fish, considering its capacity to assess the presence of neutralizing antibodies (Skliris et al., 1999; Tanaka et al., 2001). Circulating antibodies can persist over one year, although a large variation in response to immunization has been observed between individuals (Breuil and Romestad 1999; Breuil et al., 2000; Grove et al., 2006, Kai et al., 2010). However, very few information on seroconversion and antibodies persistence during natural outbreaks are available. Due to this gap of knowledge, so far the detection of specific antibodies has not been considered as a possible routine screening method (Anonymous, 2013).

6. Control strategies

A number of promising experimental vaccines against VNN have so far been tested and administered by intramuscular or intraperitoneal injection in different fish species. These included inactivated whole virus (Kai and Chi, 2008; Kai et al., 2014; Pakingking et al., 2010; Yamashita et al., 2005), recombinant capsid protein (Husgaro et al., 2001; Tanaka et al., 2001; Thiery et al., 2006), virus-like particles (VLPs) produced in different systems (Lai et al., 2014; Liu et al., 2006), as well as recombinant DNA (Vimal et al., 2014).Good results have also been obtained with the use of a live vaccine in grouper reared under controlled conditions (i.e. low temperature) (Nishizawa et al., 2012; Oh et al., 2013). However, due to the ability of betanodavirus to cause persistent infection, this approach does not seem the most suitable to guarantee safety on a long-term basis.

Larval and juvenile developmental stages are the more susceptible to the disease, but they are almost impossible to inject with a vaccine. To overcome this problem, vaccine formulation for bath administration has been tested in experimental conditions (Kai and Chi, 2008; Kai et al., 2014; Nishizawa et al., 2012). Despite some successful experiments, available data are not sufficient to confirm the efficacy of this strategy. The oral administration of immunizing products has recently been described (Lin et al., 2007). Potentially, oral vaccines may be administered to larvae as soon they start eating live pray (e.g. *Artemia salina* and rotifers) (Lin et al., 2007), micronized feed or protected with nanoparticles (Vimal et al., 2014). This method reduces the manipulation-related level of stress.

Unfortunately, nowadays commercially available vaccines against betanodavirus are not available in the market. This makes it very difficult to control the spread of the disease, which is likely to expand in highly densely fish farmed area (i.e. the Mediterranean Basin and south-eastern Asia). In absence of an efficient patented vaccine, the best way to prevent the introduction or the spread of betanodavirus in a farm is to comply with strict biosecurity measures. As a matter of fact, biosecurity must play a primary role in hatcheries where the introduction of VNN may have devastating consequences. In particular, the disinfection of inlet water, the division of the hatchery in sectors with dedicated personnel and equipment, the periodic disinfection of tanks and equipment, quarantine and accurate tests of newly introduced broodstock and control of frozen and live feedseem to be crucial (Maltese and Bovo, 2007; Munday et al., 2002). Clearly, these measures cannot be applied to open environments such us lagoons and sea cages. Nevertheless, in these environments biosecurity precautions such as fast disposal of carcasses, reduction of stock densities and feeding rate, regular fallowing as well as reduction of stress practices may be helpful to reduce the losses caused by infection.

7. Aims of the thesis

The Mediterranean basin is one of the most exploited areas for mariculture, and VNN has been recently recognized as one of the major issues for the expansion of aquaculture in this region. Despite the wide diffusion of VNN and its severe economic impact, there is an urgent need to invest in research at different levels, as limited knowledge is available on the disease itself and its aetiological agent.

In this thesis, a multi-disciplinary approach has been applied to study betanodaviruses currently circulating in the Mediterranean basin, with particular emphasis on the newly emerged reassortants. The scope of the thesis is to generate basic knowledge and develop suitable tools which can contribute to improve the control strategies against VNN. In detail, the objectives of the thesis are:

- To better understand betanodavirus epidemiology in the Mediterranean area in both wild and farmed fish (**Chapters 2 and 3**);
- To develop a database to share genetic and epidemiological data on betanodavirus disease outbreaks (**Chapter 4**);

- To implement the molecular and the serological diagnosis for betanodavirus (**Chapters 5 and 6**)
- To genetically and phenotypically characterize viral genotypes currently circulating in the Mediterranean region, with special attention to betanodavirus immunoreactivity and temperature sensitivity (**Chapters 6 and 7**);
- To assess the pathogenicity of different betanodavirus genotypes *in vivo* and in response to temperature (**Chapter 8**).

Chapter 2

Viral Encephalopathy and Retinopathy in groupers (*Epinephelus*spp.) in southern Italy: a threat for wild endangered species?

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CASE REPORT Open Access

Viral Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for wild endangered species?

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Abstract

Background: Betanodaviruses are the causative agents of Viral Encephalopathy and Retinopathy (VER). To date, more than 50 species have proved to be susceptible and among them, those found in genus *Epinephelus* are highly represented. Clinical disease outbreaks are generally characterized by typical nervous signs and significant mortalities mainly associated with aquaculture activities, although some concerns for the impact of this infection in wild fish have been raised. In this study, the authors present the first documented report describing an outbreak of VER in wild species in the Mediterranean basin.

Case presentation: In late summer - early winter 2011 (September-December), significant mortalities affecting wild Dusky grouper (*Epinephelus marginatus*), Golden grouper (*Epinephelus costae)* and European sea bass (*Dicentrarchus labrax*) were reported in the municipality of Santa Maria di Leuca (Northern Ionian Sea, Italy). The affected fish showed an abnormal swimming behavior and swollen abdomens. During this epizootic, five moribund fish showing clear neurological signs were captured and underwent laboratory investigations. Analytical results confirmed the diagnosis of VER in all the specimens. Genetic characterization classified all betanodavirus isolates as belonging to the RGNNV genotype, revealing a close genetic relationship with viral sequences obtained from diseased farmed fish reared in the same area in previous years.

Conclusion: The close relationship of the viral sequences between the isolates collected in wild affected fish and those isolated during clinical disease outbreaks in farmed fish in the same area in previous years suggests a persistent circulation of betanodaviruses and transmission between wild and farmed stocks. Further investigations are necessary to assess the risk of viral transmission between wild and farmed fish populations, particularly in marine protected areas where endangered species are present.

Keywords: Viral Encephalopathy and Retinopathy, Betanodavirus, Neurological signs, Wild fish, *Epinephelus* spp.

Background

Viral Encephalopathy and Retinopathy (VER) is a viral infectious disease affecting more than 50 marine fish species worldwide and it is considered one of the most important threats for mariculture in the Mediterranean.

Fish affected by VER generally show clear clinical signs such as anorexia, skin darkening, blindness and abnormal swimming behaviour. Typical histological lesions include cellular vacuolation, necrosis and neuronal degeneration in the central nervous system (CNS).

The aetiological agent of VER is comprised in the family *Nodaviridae*, genus *Betanodavirus*, and it is an icosahedral, non-enveloped viral particle of about 25 nm in diameter, with a genome made of two single-stranded positive-sense RNA molecules. The RNA1 (3.1 Kb) and the RNA2 (1.4 Kb) genetic segments encode the RNA-dependant RNA-polymerase (RdRp) and the coat protein respectively [1], while the RNA3 subgenomic transcript originating from the RNA1 molecule (0.4 Kb) is translated into protein B2, which

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is involved in viral intra-cellular replication mechanisms [2]. On the basis of the phylogenetic analysis of the T4 variable region within the RNA2 segment, five genotypes have been described to date: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), red-spotted grouper nervous necrosis virus (RGNNV) and turbot nervous necrosis virus (TNNV) [3,4]. Recently, betanodavirus reassortant strains possessing genome segments belonging to parental RGNNV and SJNNV have been also described [5-7].

The genus *Epinephelus* is considered highly susceptible to betanodavirus infection. Wild and farmed groupers have been frequently involved in natural outbreaks of the disease in Asian countries: i.e. mortality caused by VER has been described in *E. fuscoguttatus, E. akaara* in Japan, *E. septemfasciatus* in Japan and Korea, *E. coioides* in Philippines, *E. awooara* in Taiwan*, E. tauvina* in Malaysia, Philippines and Singapore, *E. moara* in Japan [8], *E. lanceolatus* and *E. malabaricus* in Taiwan [9] and *E. marginatus* in Taiwan[10].

In the Mediterranean Sea, VER represents a major limiting factor for the development of *E. marginatus* rearing activity both for feeding consumption [11] and preservation programs [12], and the virus has been sporadically detected in wild *E. aeneus* [13] and *E. costae* [5]. Several epidemiological surveys highlighted the presence of betanodavirus in farmed animals and wild asymptomatic fish stocks other than groupers in the Mediterranean basin [5,7,12-15]; thus underlying the widespread of this virus in this area. Although rumors of groupers mortality in several areas of the Mediterranean Basin, such as Sicily, Corsica, the Balearic Islands and other parts of the Spanish coast, Algeria, Tunisia and Greece, have circulated in the recent past, to the authors' knowledge a description of the phenomenon, as well as the demonstration of the causative agents have never been clearly documented.

From an ecological and economic point of view, *Epinephelus spp*. is considered one of the most relevant fish genus for the Mediterranean sea; moreover, some grouper species, i.e. *E. marginatus*, are considered endangered [16], and their survival is safeguarded in marine protected areas (MPA) where angling and scuba diving are strictly regulated.

In this paper an outbreak of severe disease and mortality associated to betanodavirus infection in wild fish, including groupers, is described.

Case presentation

The outbreak started in late September 2011 and lasted until the beginning of December 2011, and was characterized by extraordinary climatic conditions. At the end of summer 2011, unusual high temperatures of the entire water column were recorded for more than 30 days. In the period between September-November 2011 the mean water temperature varied from 25.99°C-17.57°C at 5 meters depth to 21.82°C-17.21°C at 35 meters depth (authors' unpublished data). Most likely, the uniform high temperature in all the water column was caused by a prolonged elevated surface pressure associated with the absence of meteorological disturbances that deepened the thermocline.

In that period, fishermen reported the presence of a high number of dead or moribund wild fish (>200 specimens) in the area of sea surrounding the municipality of Santa Maria di Leuca, (Northern Ionian Sea, Italy (Figure 1). At the same time, scuba divers related the occurrence of dead groupers in rock caves and, notably, videotaped a moribund specimen characterized by an abnormal swimming behaviour (Figure 2; Additional file 1). The occurrence of mortality in wild fish was widely discussed by local media and reported as being an unusual phenomenon.

Five clinically affected wild adult fish, belonging to three different species, were caught by local veterinary authorities and submitted to the laboratory for analysis, namely n=3 golden grouper (*Epinephelus costae*), n=1 dusky grouper (*E. marginatus*) and n=1 European sea bass (*Dicentrarchus labrax*). At the time of capture, all fish displayed an abnormal swimming behaviour, loss of swim bladder control, blindness and skin erosion in the head region. Necropsy and bacteriological analysis on tissue specimens from kidney, spleen and central nervous system (CNS) were carried out according to standard procedures. Spleen, kidney and CNS from diseased fish were taken with sterile loop and plated onto Blood Agar medium. The plates were incubated at room temperature for 48 h and checked daily.

Virological investigations were performed by means of Real time PCR [17] and virus isolation [18] on a total of 17 samples collected from the central nervous system (CNS), and, whether available, retina, anterior kidney, blood and gonads (Table 1). According to Panzarin et al. 2012 [4], for each specimen sequencing and phylogenetic analysis were performed on viral strains isolated from the brain, with the exception of sample 425.1 isolated from the retina. Using the MEGA 4 package [19], sequences from both genes (867 bp-long RNA1 fragment and 504 bp-long RNA2 fragment) were aligned and compared to sequences publicly available in GenBank, as well as with unpublished sequences of viruses isolated from fish reared in sea cage farms during previous outbreaks of VER in the same area (Table 2). Phylogenetic trees were developed for both genetic segments using the neighbour-joining (NJ) method with 1000 bootstrap re-samplings. Nucleotide similarities were also determined.

Results and discussion

VER has been reported in the Mediterranean basin since 1991 and has proved to be a major problem for reared

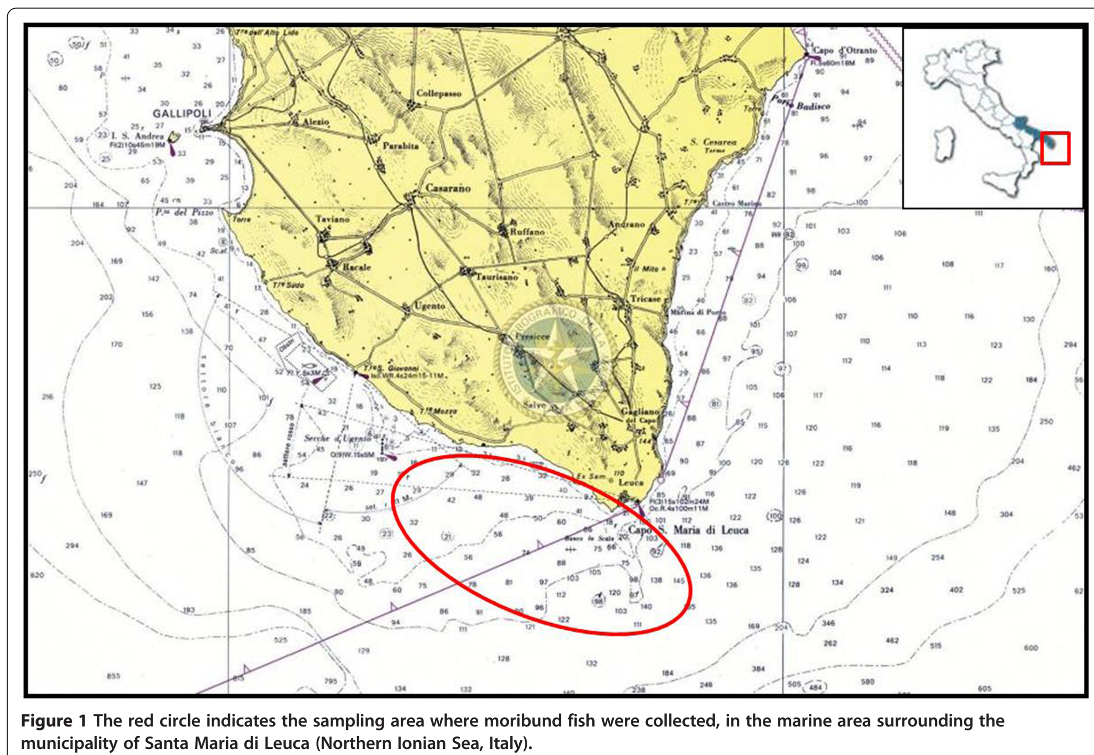

Figure 1 The red circle indicates the sampling area where moribund fish were collected, in the marine area surrounding the municipality of Santa Maria di Leuca (Northern Ionian Sea, Italy).

marine fish [11,15,20,21]. Nevertheless, subsequent epidemiological investigations highlighted the presence of betanodavirus also in several apparently healthy wild species [5,7,14]. Some sporadic cases of mortality in wild groupers likely associated to VER were reported in the Mediterranean basin, but so far only few laboratory-confirmed cases have been described [13,22]. Currently, detailed information on the occurrence of the disease in wild stocks in this area is missing.

All five fish collected for primary necropsy and further focused laboratory investigation showed: erosions, ulcers and scale loss in the head region, swim bladder hyperinflation, corneal opacity; hyperhaemia with spread blood vessel congestion of CNS was also observed (Figure 2).

Bacteriological examinations for the detection of common systemic bacterial pathogens yielded negative results. Virological analysis revealed that 15 out of 17 samples tested positive for betanodavirus detection by real time PCR, 13 of which were confirmed by virus isolation in cell culture (Table 1).

The phylogenetic trees inferred for the polymerase (RNA1) and the coat protein (RNA2) partial genes consistently grouped all the isolates within the RGNNV genotype, the most common in the Mediterranean sea (Figure 3). Sequences related to strains isolated from golden grouper (396.3.2011, 425.2.2011, 496.1.2011) showed high nucleotide similarity (99.8-100% for RNA1; 99.4-100% for RNA2) with a panel of betanodavirus strains isolated during clinical outbreaks occurred in previous years (2006–2009) in European sea bass reared in neighbouring fish farms. Lower nucleotide similarity (99.7-99.8% for RNA1 and 99.1-99% for RNA2) was observed between sequences related to viral strains detected in farmed fish and samples 385.1.2011 and 424.1.2011. Interestingly, viral strains isolated from dusky grouper and European sea bass (samples 385.1 and 424.1, respectively) were identical and phylogenetically distinguishable from the golden grouper betanodaviruses. Overall, similarity ranges among betanodavirus strains herein characterized were 100–99.7% and 100-99% for RNA1 and RNA2, respectively.

The mortality rate of an outbreak in wild fish is generally extremely difficult to evaluate since several variables which are quite difficult – if not impossible – to measure should be considered. For instance, it is very hard to assess the extent of the outbreak if a great number of dead fish inside rock caves in the area is found, or in case moribund fish on the sea surface (due to swim bladder inflation) are collected by fishermen or captured by other fish or birds.

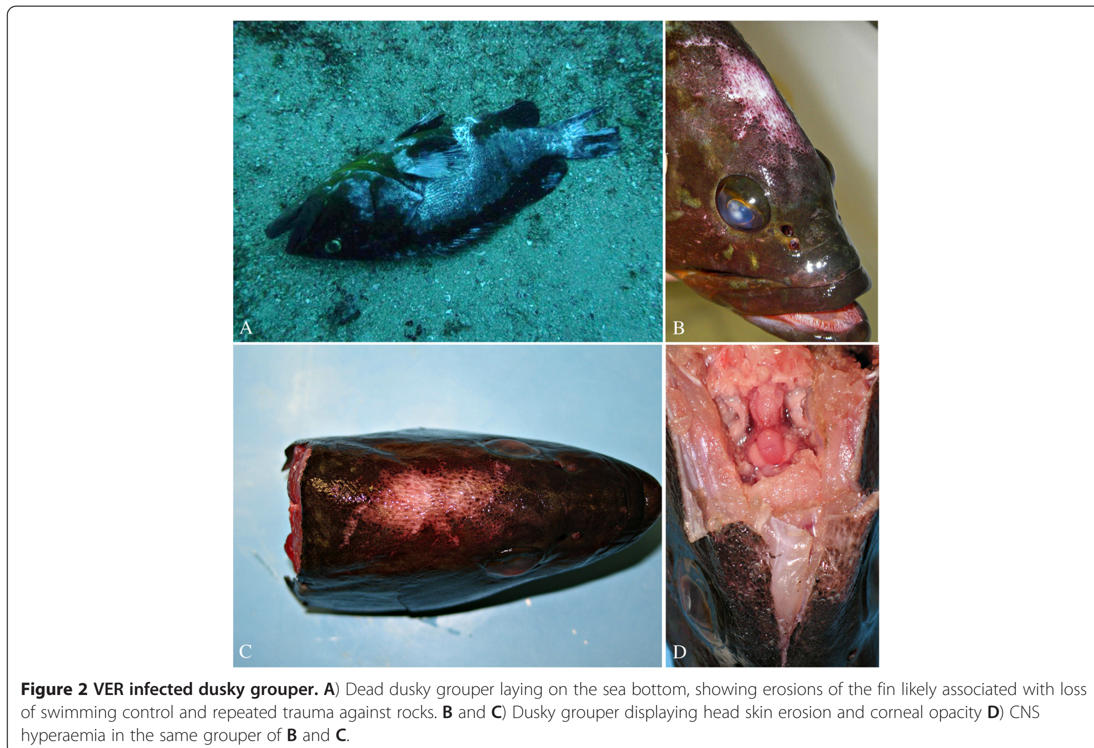

Figure 2 VER infected dusky grouper. A) Dead dusky grouper laying on the sea bottom, showing erosions of the fin likely associated with loss of swimming control and repeated trauma against rocks. **B** and **C**) Dusky grouper displaying head skin erosion and corneal opacity **D**) CNS hyperaemia in the same grouper of **B** and **C**.

However, the estimated number of dead fish (more than 200) reported by local authorities, fishermen and diving centres is an indication of how severely this outbreak affected wild stocks.

The high water temperature has often proved to be an environmental factor associated with the development of clinical disease within aquaculture activities [23]. Thus, given the thermal anomalies characterising the water column during the outbreak, the onset of clinical disease in wild populations can be ascribed to this condition.

Aquaculture facilities, consisting in 12 floating cages, are located approximately 15–20 kilometres North-East from the place of the epidemic. In these cages European sea bass (*D. labrax*) Gilthead sea bream (*Sparus aurata*) and some other minor species are reared and fattened for human consumption with a production of 400 tons per year. Considering that data on the health condition of the farmed fish during the outbreak in the wild species herein described were not available, viral sequences belonging to strains isolated in the area from previous years in farmed fish were included in the phylogenetic study.

To date, only few data are available regarding viral transmission between marine wild and farmed fish [4] and it is still unknown which is the direction of the viral

Table 1 Samples collected from clinically affected golden grouper, dusky grouper and European sea bass, and molecular and virological analytical results

Samples ID	Host species	Sample matrix	Real Time PCR (CP)	Cell culture isolation
385.1	*E. marginatus*	CNS	+ (9.48)	+
385.2		Retina	+ (15.23)	+
385.3		Anterior kidney	+ (28.43)	+
386.3		Blood	-	-
396.3	*E. costae*	CNS	+ (17.59)	+
396.4		Optical nerve	+ (26.93)	+
424.1	*D. labrax*	CNS	+ (14.34)	+
424.2		Retina	+ (15.06)	+
424.3		Optical nerve	+ (21.69)	+
424.4		Anterior kidney	+ (28.99)	-
424.5		Gonads	-	-
425.1	*E. costae*	Optical nerve	+ (32.50)	+
425.2		Retina	+ (15.33)	+
425.3		Anterior kidney	+ (19.37)	+
496.1	*E. costae*	CNS	+ (16.8)	+
496.2		Retina	+ (14.5)	+
496.3		Anterior kidney	+ (25.3)	-

Table 2 Data collected in this study on isolates included in the phylogenetic analysis and related GenBank accession number

Strain	Year	Host	Clinical sings	Status	GenBank accession no.	
					RNA1	RNA2
332.2	2006	*D. labrax*	Not available	Farmed	JN189872	JN190012
327.7	2008	*D. labrax*	Present	Farmed	JX290528	JX290538
352.1	2008	*D. labrax*	Present	Farmed	JX290526	JX290540
549.13	2008	*D. labrax*	Present	Farmed	JX290527	JX290536
2.5	2009	*D. labrax*	Present	Farmed	JX290524	JX290537
2.6	2009	*D. labrax*	Present	Farmed	JX290525	JX290539
469.1	2010	*D. labrax*	Present	Farmed	JX290529	JX290541
469.2	2010	*D. labrax*	Present	Farmed	JX290530	JX290542
385.1	2011	*E. marginatus*	Present	Wild	JX290520	JX290532
396.3	2011	*E. costae*	Present	Wild	JX290521	JX290533
424.1	2011	*D. labrax*	Present	Wild	JX290522	JX290534
425.2	2011	*E. costae*	Present	Wild	JX290523	JX290535
496.1	2011	*E. costae*	Present	Wild	JX290531	JX290519

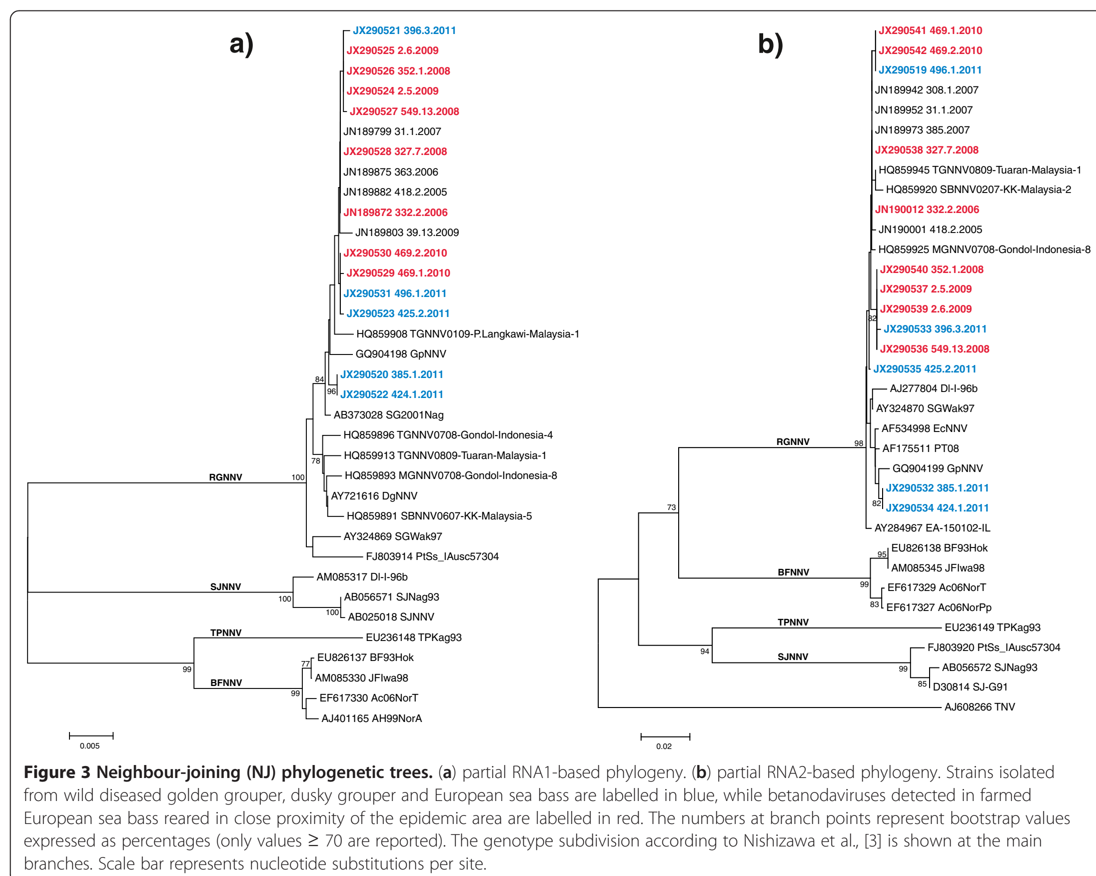

Figure 3 Neighbour-joining (NJ) phylogenetic trees. (**a**) partial RNA1-based phylogeny. (**b**) partial RNA2-based phylogeny. Strains isolated from wild diseased golden grouper, dusky grouper and European sea bass are labelled in blue, while betanodaviruses detected in farmed European sea bass reared in close proximity of the epidemic area are labelled in red. The numbers at branch points represent bootstrap values expressed as percentages (only values ≥ 70 are reported). The genotype subdivision according to Nishizawa et al., [3] is shown at the main branches. Scale bar represents nucleotide substitutions per site.

flow (i.e. wild to farmed fish or viceversa) -possibly the flow is bidirectional- [22]. Regarding this specific case, the high genome similarity among viruses isolated in different years in wild and farmed fish from the same area is highly suggestive of a persistent circulation of betanodaviruses and transmission between wild and farmed stocks. Due to a lack of monitoring plans in wild fish populating that area, further studies are required to assess the risk of spreading pathogen(s) from/to the wild.

Conclusions

In the present study we described a severe outbreak of VER in three different wild carnivore fish species, namely dusky grouper, golden grouper and European sea bass in the Northern Ionian Sea, Italy. To the authors' knowledge, this is the first documented report describing a disease outbreak in wild species in the Mediterranean basin, with a documented description of clinical signs associated to the isolation and characterization of the causative agent. These results are consistent with the report by Gomez and colleagues [24] describing for the first time an outbreak of VER in wild adult *E. akaara* in Japan, and confirm the high susceptibility of the genus *Epinephelus* to this pathogen.

The observation of this outbreak possibly represents a starting point for further investigations aiming to evaluate pathogens transmission between farmed and wild fish and to assess the risk of viral exchange between wild and farmed fish populations. The potential transmission of the disease from farm to wild or vice versa must be seriously taken in consideration, with particular reference to the management of marine protected areas (MPA) and fish preservation projects.

Additional file

Additional file 1: Dusky grouper (*E. marginatus*) moribund specimen video-recorded in October 2011 from a scuba diving instructor in Santa Maria di Leuca. This fish, observed 30 mt deep, displays an abnormal swimming behaviour and blindness. Samples 385.1 (CNS), 385.2 (retina), 385.3 (anterior kidney) and 386.3 (blood) were collected from this specimen and subjected to laboratory investigations. Betanodavirus was detected by Real Time PCR and viral isolation in cell culture (SSN-1) in all the samples, with the exception of sample 386.3 (blood) which yielded negative results.

Abbreviations

VER: Viral encephalopathy and retinopathy; CNS: Central nervous system.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

NV wrote the paper; PP designed and coordinated the study and together with PT and AT collected samples from the field and information about the mortality event of fish; EC and VP performed virological and phylogenetic analysis respectively; AT supervised the laboratory activities and helped to draft the manuscript; CT and GC have given final approval of the version to be published. All authors read and approved the final manuscript.

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Chapter 3

Outbreak of viral nervous necrosis in endangered fish species *Epinephelus costae* and *E. marginatus* in northern Tunisian coasts

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Short communication

Outbreak of Viral Nervous Necrosis in Endangered Fish species *Epinephelus costae* and *E. marginatus* in Northern Tunisian Coasts

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ABSTRACT—In this study, we report outbreak of viral nervous necrosis (VNN) in wild *Epinephelus* species, which are of an endangered fish group, in different Tunisian coastal areas in 2012. Seven fish of *E. marginatus* and *E. costae* caught at dead or moribund condition were investigated. Betanodavirus was detected in the brain and retinal tissues of all fish by RT-PCR and at high infective titers ($10^{6.0-8.8}$ $TCID_{50}$/g) in five of seven fish. Sequence and phylogenic analyses of the viral genes revealed that the viruses belonged to RGNNV genotype and were closely related to some previously reported Mediterranean betanodavirus strains, suggesting virus exchanges among different fish populations in the Mediterranean Sea.

Key words: betanodaviruses, disease outbreak, *Epinephelus* spp., phylogeny, RGNNV

Viral nervous necrosis (VNN) is a major concern for marine aquaculture (Munday *et al.*, 2002). Similarly, VNN represents a menace to wild fish populations, affecting numerous species of nine distinct orders (Panzarin *et al.*, 2012). Diseased fish present an uncoordinated swimming behaviour as a consequence of lesions occurring in the central nervous system. In larval and juvenile stages, this disease can cause up to 100% mortality and in adults, significant mortality was reported (Bovo *et al.*, 1999; Munday *et al.*, 2002).

VNN is caused by betanodaviruses, non-enveloped viruses belonging to the family *Nodaviridae.* Betanodaviruses have a single-stranded positive-sense RNA genome divided into two segments: RNA1 (3.1 kb) and RNA2 (1.4 kb) coding for a RNA-dependent RNA polymerase and a coat protein respectively (Mori *et al.*, 1992). On the base of phylogenetic analysis of the RNA2 segment, five genogroups have been described: red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) and turbot nervous necrosis virus (TNNV) (Nishizawa *et al.*, 1997; Johansen *et al.*, 2004). However, NNV genetic diversity is more complex since many reassortant viruses bearing an RNA1 segment related to a specific genotype and an RNA2 segment from another genotype have been described. Thus the combined characterization of RNA1 and RNA2 segments is recommended (Panzarin *et al.*, 2012).

The genus *Epinephelus*, one of the most relevant fish genus, is highly susceptible to betanodavirus infection. Numerous VNN outbreaks have been reported in farmed and wild *Epinephelus* spp. in Asia (Munday *et al.*, 2002). In the Mediterranean sea, VNN outbreaks in *Epinephelus* spp. was first reported in 1999 in the natural marine reserve of Ustica, Italy (Marino and Azzurro, 2001) and recently in 2011 in the South of Italy (Vendramin *et al.*, 2013a) and the North-eastern of Algeria (Kara *et al.*, 2014). RGNNV isolates were also found in such groupers in 2001, 2002 and 2009 in Italy and Greece (Panzarin *et al.*, 2012).

In the Mediterranean Sea, the genus *Epinephelus* is of great interest from an ecological and economic point of view. Among these species, *E. marginatus,* which is heavily exploited, is safeguarded and included in the Red list of marine fishes (Zabala *et al.*, 1997). Particularly, in Tunisia, groupers are represented by six species: *E. aeneus, E. caninus, E. costae, E. haifensis, E. marginatus* and *Mycteroperca rubra* (Bradai *et al.*, 2004). Notably, standings and mortalities of wild groupers, such *Epinephelus* spp., were reported in the Northern regions during the last years. Betanodavirus RGNNV strains were isolated from farmed fishes since 1992 (Thiéry *et al*., 2004) and, recently in 2011, we demonstrated the spread of RGNNV strains among farmed fishes along the Tunisian coasts (Haddad-Boubaker *et al.*, 2013). However, the presence of betanodavirus in wild fish stocks has never been officially described so far.

The present study reports the occurrence of severe disease and mortality of wild *Epinephelus* species in association with betanodaviruses in northern coasts of Tunisia (Fig. 1).

Material and Methods

In 2012, in the period of August to November, severe mortalities of wild dusky groupers *E. marginatus* and golden groupers *E. costae* in northern Tunisian coasts (Tabarka, Cap Negro, Cap Serrat, Kef Abed, Bizerte, Cap Bon and Kelibia were documented by fishermen and scuba divers (Fig. 1). More than 250 specimens presented neurologic signs or were recovered stranding on the beach. A total of eight samples were collected from September to November (Table 1):

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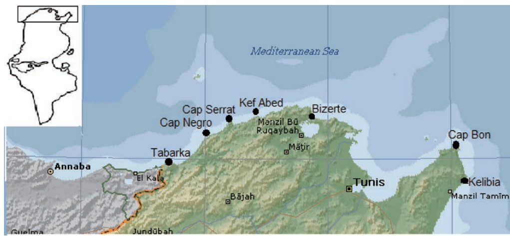

Fig. 1. Geographical distribution of sites where moribund groupers were observed.

seven wild *Epinephelus* specimens sampled in Tabarka, Bizerte, Kelibia regions (Fig. 1) and one farmed European seabass *Dicentrarchus labrax* specimen obtained during an outbreak in the Sahel region (Eastern Tunisian coast) (Table 1). *Epinephelus* specimens were identified as *E. marginatus* (n = 4) and *E. costae* (n = 3). Six of them were caught by fishermen and exhibited opaque eyes, while one moribund specimen was collected alive by scuba diver during an exploration of Tabarka region in the first of November (Table 1). The moribund fish (T2.12) presented erratic swimming behavior with loss of equilibrium and, sometimes, sank to the bottom and then floated to the surface again. The sea surface temperature was 24°C at the time of capture. The caught specimens were obtained in September (T1.12, B1.12, K1.12), October (T3.12, B2.12) and November (T4.12) at 26–27°C, 24–25.5°C and 22°C sea surface temperature respectively. Concerning the *D. labrax* specimen, the fish was collected at the first of September at 27°C sea surface temperature.

The total length and weight of each specimen were recorded. Examination and dissection of fish were focused on the observation of swim bladder and search of parasites in the gills and digestive tract. At autopsy, brain and retinal tissues were sampled. Portions of brain obtained from specimens T1.12, B2.12 and K1.12, caught respectively from Tabarka, Bizerte and Kelibia regions, were conserved for histopathological examination.

Virus isolation was assessed on striped snakehead fry cell line (SSN-1) (Frerichs *et al.*, 1996). Briefly, SSN-1 cells were propagated at 29°C in Leibovitz medium (L-15) (Sigma) supplemented with 10% of fetal bovine serum, penicillin (100 I.U./mL) and streptomycin (0.1 mg/mL). The samples of brain and retinal tissues were separately homogenized with a mortar and pestle and diluted 1:10 (w:v) in L-15 medium containing penicillin (200 I.U./mL), streptomycin (0.2 mg/mL) and kanamycin (0.2 mg/mL). The homogenates were centrifuged at 3,000 × *g* for 15 min (4°C) and the supernatants were either immediately used or stored at –80°C. Virus isolation was assessed at 22–27°C, according to the water temperature during fish collection. Infectivity viral titers, expressed as tissue-culture infective dose ($TCDI_{50}$/mL), were calculated as described by Reed and Muench (1938).

For molecular detection, RNA extraction was performed by the NucleoSpin RNA II kit (Macherey-Nagel) on the tissue homogenates. A segment of 170 bases in the RNA1 gene was amplified using oPVP154 and oPVP155 primers and the One-Step RT-PCR kit (QIAGEN) (Haddad-Boubaker *et al.*, 2013).

For genetic characterization, products of 680 bases in the RNA2 segment and 930 bases in the RNA1 segment were obtained using the oPVP88/oPVP111 (Bigarré *et al.*, 2010) and the VNNV5/VNNV6 primer sets (Toffolo *et al.*, 2007) respectively and the One-Step RT-PCR kit (QIAGEN).

The phylogenetic analysis of the obtained RNA1 and RNA2 sequences and relevant sequences available in GenBank was conducted using the maximum-likelihood method available in the MEGA 5.1 package. Robustness of individual nodes was confirmed with 1,000 bootstrap replicates. The sequences generated in this study were submitted to GenBank (Table 1).

Results and Discussion

The examination of the digestive tract exhibited a total vacuity (100%) and no signs of parasitism. A hyperinflated swim bladder was observed in five samples (T1.12, T2.12, T3.12, B2.12 and K1.12).

Histopathological examination of the three samples of brain (T1.12, B2.12 and K1.12) revealed the presence of severe cytoplasmic vacuolation with consistent nuclear pyknosis suggesting the involvement of betanodavirus in the mortality.

Molecular investigation showed positive PCR results for all the samples confirming the presence of betanodavirus. For five samples, positive results were also confirmed by virus isolation (T4.12 and B1.12 samples were negative): virus infective titers in the brain

Table 1. Details of investigated fish specimens and related GenBank accession numbers

Specimen	Species	Geographic origin	Weight (kg)	Total length (cm)	Clinical signs	Virus Titer ($TCID_{50}$/g)		Accession number	
						Brain	Retina	RNA1	RNA2
T1.12	*E. marginatus* (w)	Tabarka	5.3	65	Opaque eyes	$10^{8.2}$	$10^{7.5}$	KF748953	KF748945
T2.12	*E. costae* (w)		6.9	74	Neurological signs, Superficial lesions	$10^{8.8}$	$10^{7.9}$	KF748949	KF748941
T3.12	*E. marginatus* (w)		3.2	59	Opaque eyes	$10^{6.9}$	$10^{6.0}$	KF748950	KF748942
T4.12	*E. costae* (w)		7.2	69	Opaque eyes	–	–	KF748951	KF748943
B1.12	*E. costae* (w)	Bizerte	4.6	64	Opaque eyes	–	–	KF748952	KF748944
B2.12	*E. marginatus* (w)		6.3	72	Opaque eyes	$10^{7.0}$	$10^{6.2}$	KF748954	KF748946
K1.12	*E. marginatus* (w)	Kelibia	5.2	66	Opaque eyes	$10^{6.4}$	$10^{6.0}$	KF748955	KF748947
S16.12	*D. labrax* (f)	Sahel region	0.15	11	No	$10^{7.3}$	$10^{7.0}$	KF748956	KF748948

(w): Wild fish; (f): Farmed Fish

and retinal tissues were $10^{6.4}$–$10^{8.8}$ $TCID_{50}$/g and $10^{6.0}$–$10^{7.9}$ $TCID_{50}$/g respectively (Table 1). Thus, high infective titers in nervous organs in addition to positive molecular results confirmed involvement of betanodavirus in the mortality for five samples (T1.12, T2.12, T3.12, B2.12 and K1.12) collected from the three Northern Tunisian regions. In the other hand, for samples T4.12 and B1.12, the absence of swim bladder inflation and cytopathic effect on SSN-1 cells may only suggest a carriage of betanodavirus.

The phylogenic analysis of the RNA1 and RNA2 sequences demonstrated that the studied viruses belong to the RGNNV genotype (Fig. 2). These viruses shared 95.2–100% and 96.2–100% nucleotide identity for the RNA1 and RNA2 segments respectively. Both topologies confirmed that strains T1.12 and B2.12 detected in *E. marginatus* specimens in two different regions are 100% identical, but differ from the other Tunisian strains herein characterized. On the base of the RNA1 and the RNA2 sequences, they were highly related to Italian strains (JX290521 and JX290531) isolated from wild *Mullus barbatus* (99.8% and 99.7% identity, respectively) and *E. costae* (99.7% and 99.5% identity, respectively) collected in 2011 as well as to a Tunisian strain (HE796793) (99.9% RNA2 identity) obtained during an outbreak in 2011. B1.12 strain originating from Bizerte showed to be highly related to samples T2.12, T3.12 and T4.12 collected in Tabarka (99.9% identity for RNA1 and RNA2, respectively). Viruses S16.12 and K1.12 detected in farmed sea bass and wild dusky grouper respectively, were highly similar to each other (99.9% identity for RNA2). Overall, high nucleotide similarities were observed between the investigated viruses and Tunisian viruses previously detected in farmed fish in 2011 (HE796793, HE796785, HE796792 and HE796795) as well as with viral isolates originating from Italy (JX290521, JX290531, JN189799, JN189952) and Greece (JN189823, JN189828, JN189980, JN189975). Taken together, these data suggest the existence of epidemiological connections between different geographic areas of Tunisia and the Mediterranean, and the occurrence of viral exchanges between reared and feral fish.

In the Mediterranean basin, several reports described the presence of betanodavirus in wild fish stocks (Ciulli *et al.*, 2007; Panzarin *et al.*, 2012; Vendramin *et al.*, 2013a). In this study, we report, for the first time, the occurrence of a severe VNN outbreak affecting a large number of *Epinephelus* specimens in different northern Tunisian regions. This mortality episode seems to become recurrent phenomenon in accordance with the augmentation of water temperature. Indeed, the investigation conducted with professional fishermen and diving clubs in the region of Tabarka showed that the mortality episodes of groupers had existed during warm season (August to November) since 1984. The phenomenon sporadically reappeared in the following years, and became recurrent with more emphasis after 2003, affecting annually a larger number of fish species (*Balistes carolinensis*, *Corvina nigra*, *Diplodus sargus*) and extending to a wider geographic area (Bizerte, Cap Serrat, Cap Negro, Kef Abed and Kelibia) (Fig. 1), although the involvement of betanodavirus in this mortality has never been officially investigated. It is generally assumed that the water temperature is a major factor for the development of clinical disease. In northern Tunisia, the average of sea surface temperatures has

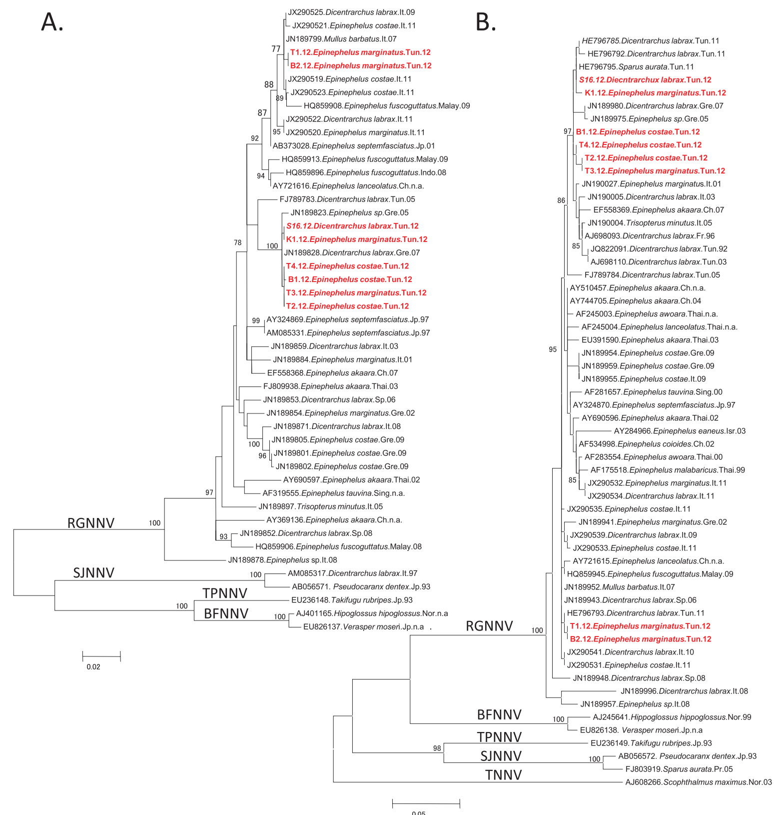

Fig. 2. Comparative phylogenetic analysis of the investigated viruses based on the partial RNA1 and RNA2 sequences: (A) Partial RNA1 sequence based tree; (B) Partial RNA2 sequence based tree. The phylogenetic tree was inferred through the Maximum Likelihood method with 1000 bootstrap replicates (only values ≥ 75 are reported at branch points). The genotype subdivision is shown at the main branches. The scale bar represent the number of substitutions per site. Tunisian viruses obtained from wild golden grouper, dusky grouper and European sea bass, in 2012, are labeled in red. n.a., not available.

increased by 0.9°C during the last 50 years, according to the Simple Ocean Data Assimilation database, and the largest rises occurred between 2000–2009, the period from which this disease has spread (data not shown). This significant increase of temperature could have disastrous consequences on ecosystems and fish species such as groupers. It can be also considered as a stimulating factor of virulence of some pathogens including betanodaviruses. Many cases of betanodavirus outbreaks have been reported at 24–30°C water temperature (Fukuda *et al.*, 1996; Chi *et al.*, 1999; Athanassopoulou *et al.*, 2003) as well as the increase of betanodavirus pathogenicity under experimental conditions (Nishizawa *et al.*, 2012; Vendramin *et al.*, 2013b). However, it is difficult to assess whether the onset of clinical disease is a consequence of the optimal temperature for virus multiplication, or it is caused by the physical stress of fish induced by elevated temperatures. Both factors may probably have a role in the apparition of clinical disease.

Our phylogenic data suggest the occurrence of viral exchange between farmed and wild fish populations as previously hypothesized by Vendramin *et al.* (2013a). Notably, in this case, Tunisian aquaculture facilities are mostly located in the eastern coasts, far from Tabarka and Bizerte regions (more than 100 km) and it is known that *Epinephelus* species are territorial, generally standing in rock caves. Thus, we speculate that the transmission of viruses might occur via vectors such as other wild fish. The contamination via food chain should also be considered since groupers are carnivore, especially piscivore. However, it is difficult to assess the origin of the infection: *Epinephelus* species can constitute a reservoir acting as a potential source of virus and at the same time farmed fish might shed high levels of virus in the environment during outbreaks, causing contamination of different wild species. More investigations are needed to evaluate the role of *Epinephelus* species in the dynamics of betanodavirus infections.

In the concern of general decline of endangered fish groupers, the control of the disease by combination of good management practices and vaccination of adults and/or fries could preserve wild fish populations by limiting viral exchanges, sustaining virus rejection in the environment and in the same time could reduce severe economic losses in aquaculture facilities.

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Chapter 4

Fishpathogens.eu/noda: a free and handy online platform for Betanodavirus targeted research and data sharing

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Fishpathogens.eu/noda: a free and handy online platform for Betanodavirus targeted research and data sharing

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Abstract

Viral nervous necrosis (VNN) is a severe neuropathological disease affecting a broad variety of finfish species worldwide. The causative agents of VNN are small viruses with a bi-segmented RNA genome known as betanodaviruses. At least four species with distinct but yet insufficiently characterized epidemiological features are recognized. The spread of VNN to an increasing number of host species, its wide geographic extent and its economical and ecological impacts justify the importance of collating as much molecular data as possible for tracing the origin of viral isolates and highlight the need for a freely accessible tool for epidemiological and molecular data sharing and consultation. For this purpose, we established a web-based specific database using the www.fishpathogens.eu platform, with the aim of collecting molecular and epidemiological information on VNN viruses, with relevance to their control, management and research studies.

Keywords: betanodavirus, database, epidemiology, Fishpathogens.eu, viral nervous necrosis (VNN).

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Introduction

Betanodaviruses are the causative agents of a severe neuropathological disease known as viral nervous necrosis (VNN) (syn: viral encephalopathy and retinopathy – VER), which is considered one of the most hazardous viral diseases for marine aquaculture worldwide. The disease is highly contagious and capable of causing mass mortalities in several fish species, particularly during the earliest developmental stages (Munday, Kwang & Moody 2002). Due to its high infectivity and its adaptability to a wide range of hosts and environmental conditions, VNN represents a serious risk for economically valuable finfish species (Grotmol *et al.* 2000; Ucko, Colorni & Diamant 2004; Chérif *et al.* 2009; Ransangan & Manin 2010) and wild fish stocks (Ciulli *et al.* 2007; Gomez *et al.* 2008; Sakamoto *et al.* 2008) including endangered fish species (Gomez *et al.* 2009; Vendramin *et al.* 2013; Kara *et al.* 2014). Up to 10 years ago, VNN was believed to be a purely marine fish disease. Nevertheless, in the last decade, the number of reports of outbreaks in freshwater fish has gradually increased (Munday *et al.* 2002; Athanassopoulou, Billinis & Prapas 2004; Bigarré *et al.* 2009; Bovo *et al.* 2011), thus enlarging the list of susceptible fish species.

Betanodaviruses are naked, icosahedral viral particles of about 25–30 nm in diameter, consisting of a bi-segmented single-stranded positive-sense

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RNA genome. The RNA1 segment (3.1 Kb) encodes the RNA-dependent RNA polymerase (RdRp) or protein A, while the RNA2 segment (1.4 Kb) encodes the coat protein (Mori *et al.* 1992). The RNA3 subgenomic transcript (0.4 Kb), originating from the 3′ terminus of the RNA1 molecule, encodes the protein B2 with a RNA-silencing suppression activity (Iwamoto *et al.* 2005). According to the phylogenetic analysis of the RNA2 segment, betanodaviruses can be divided into four species, namely striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.* 1997). A fifth genotype, the turbot nervous necrosis virus (TNNV), awaits its classification in the genus (Johansen *et al.* 2004). Overall, sequence analysis of the RNA1 genetic segment identified the same genetic clustering as for the RNA2 analysis. However, discrepancies between the phylogenies of the coat protein gene and the RdRp gene have led to the identification of genetic reassortment events between different betanodavirus genotypes in several fish species. In particular, one betanodavirus strain harbouring the RNA1 of a SJNNV and the RNA2 of a RGNNV (i.e. SJNNV/RGNNV) was detected in sea bass specimens, sampled in the northern Adriatic sea (Toffolo *et al.* 2007). Conversely, a reassortant virus possessing the RGNNV-RNA1 and the SJNNV-RNA2 (i.e. RGNNV/SJNNV) was isolated from gilthead sea bream and Senegalese sole sampled in Portugal and Spain (Olveira *et al.* 2009). Later on, it was reported that the RGNNV/SJNNV reassortants actually spread also in Croatia, Cyprus and Greece and that intragenotype genetic reassortment events also occurred between RGNNV strains (Panzarin *et al.* 2012).

While the four betanodaviruses species have been shown to have optimal temperature ranges and, accordingly, peculiar geographic distributions (Panzarin *et al.* 2014), information about the properties of reassortant strains as well as the influence of genetic exchange on viral phenotype and ecology is still scarce. So far, only a few papers investigate the effect of reassortment on the features of fish nodaviruses. Panzarin *et al.* (2014) showed that the genetic type of the RNA1 molecule was the major molecular determinant regulating the level of viral replication *in vitro* in response to the incubation temperature. On the other hand, the RNA2 segment seems to determine betanodavirus host specificity as demonstrated by using infectious clones of reassortants (Iwamoto *et al.* 2004; Ito *et al.* 2008). To date, most of the sequences available in GenBank are from the RNA2 molecule. However, because of a substitution rate higher than expected for RNA1 and the existence of reassortants with putative specific biological features, the characterization of both RNA1 and RNA2 genetic components is highly recommended for the classification of newly detected viral strains (Panzarin *et al.* 2012; He & Teng 2014). Furthermore, considering the continuous dissemination of betanodaviruses, for instance by short- and long-distance fish trade, it is of primary importance to collect both genetic data and field information to help epidemiologists and stakeholders to track the origin of new disease outbreaks.

Fishpathogens.eu is a website developed and maintained by the European Union Reference Laboratory for Fish Diseases (EURL-FISH). It was launched in June 2009 focusing on viral haemorrhagic septicaemia virus (VHSV) (Jonstrup *et al.* 2009) and expanded to also include infectious hematopoietic necrosis virus (IHNV) in 2010 (Jonstrup *et al.* 2010). Today, the two databases contain information on 755 and 92 isolates and 414 and 84 sequences of VHSV and IHNV, respectively, with 117 registered users, and have proven to be valuable tools for those involved in the field of fish virology. It is possible and indeed advisable to include other fish pathogens as well. In this paper, we present the extension of FishPathogens.eu to also comprise betanodaviruses.

Overview of the betanodavirus database

The betanodavirus web platform provides a searchable database including both epidemiological and molecular information (Fig. 1a). All included reports are peer-reviewed by pathogen experts. Epidemiological information is added as an 'isolate report', to which one or more 'sequence reports' containing related molecular data can be linked. The platform allows users to upload sequences of one or both genetic segments, with different lengths. However, to harmonize input data and assist users in sequencing field strains, the authors report a non-exhaustive list of published primer sets suitable for betanodavirus genotyping (Fig. 2).

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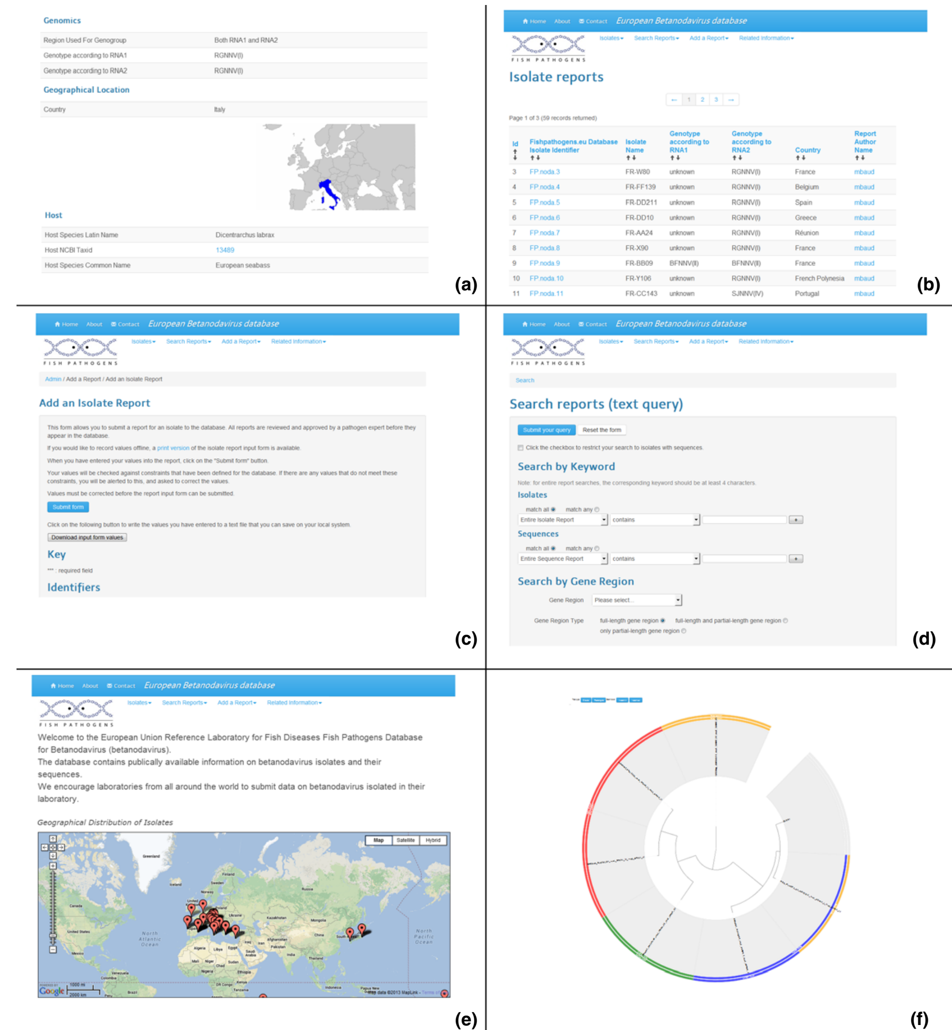

Figure 1 Screenshots taken from Fishpathogens.eu/noda show examples of the information that can be extracted from the database. (a) An example of some of the information that is available in the individual isolate and sequence reports. (b) An example of how isolate reports can be browsed and selected according to various parameters. Retrieved information can be downloaded in an Excel-compatible format. (c) An easy-to-use interface makes it easy to add new isolate and sequence reports to the database. (d) An example of some of the lists of parameters that can be used to define a search in the database. (e) Google Maps™ can be used to visualize the distribution of isolates or to define search results to certain geographic regions. (f) An example of a phylogenetic tree created using neighbour joining by the sequence matcher function.

All data stored in the database can be browsed manually (Fig. 1b). New reports can be easily added by filling out the appropriate forms on the website (Fig. 1c), and laboratories worldwide are encouraged to do so in order to increase the coverage of the database. Information on how to use the platform is also available at the website. The EURL-FISH can provide assistance for new users.

The betanodavirus database was initialized with a set of 58 isolates and 106 sequences. Several search functions are available, including a text-based search based on a number of criteria

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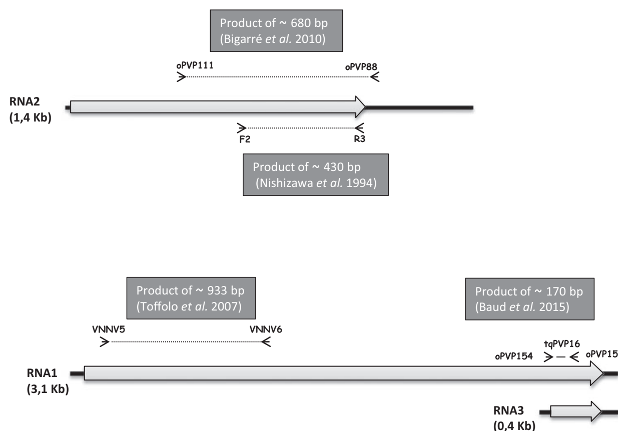

Figure 2 Primer sets recommended for betanodavirus sequencing and molecular characterization.

including (i) genotype, (ii) host, (iii) aquatic environment, (iv) time slot or (v) geographic location (Fig. 1d). It is also possible to search for isolates geospatially using Google Maps™. For convenience, a BLAST-based search function allows retrieval of all available sequences from any part of the genome based on a target sequence defined by the user.

Besides search, the database also provides mapping tools based on Google Maps™ and Google Earth™ to visualize the geographic distribution of retrieved isolates (Fig. 1e). A new feature of Fishpathogens.eu now allows constraining the number of returned isolates by manually picking out the strains of interest from a search result, making it easier to optimize the research to the needs of the user. A unique identifier for each isolate allows for easy access to and identification of specific isolates and their coupled sequence reports. Notably, retrieved information can be exported to a spreadsheet and thereby be imported to other programs whenever required. Both nucleotide and protein sequence information can be downloaded in FASTA format and imported directly to the most common sequence alignment programs.

Another function that was recently developed enables the user to compare a query sequence against a collection of nucleotide sequences available in the database. While the data set for comparison can be defined directly by the users, according to their needs, suggested and reference sets of sequences are provided for broad typing and identification. Sequence comparison is achieved by FASTA matching (Pearson & Lipman 1988) or by alignment and subsequent phylogenetic reconstruction through neighbour joining (NJ), with results visualized as a table of comparative information (e.g. nucleotide overlap, number of matches, similarity) or a drawing of the phylogenetic tree, respectively (Fig. 1f).

This database has been developed using the LAMP (Linux, Apache, MySQL and PHP) stack, a package of free, open source software. Sequence alignment is done using Bl2seq (available from NIH at ftp://ftp.ncbi.nlm.nih.gov/blast//executables) and Mafft (available from http://mafft.cbrc.jp/alignment/software/). Phylogenetic reconstruction is done with Quickjoin (available from http://www.daimi.au.dk/~mailund/quick-join.html), and phylogenies are drawn with JPhyloSVG (available from http://www.jsphylosvg.com/). The fishpathogen.eu/noda database can be viewed using recent versions of common web browsers, including Internet Explorer, Safari, Firefox and Opera. The EURL-FISH will be responsible for updating the database.

Accessibility and availability

The database is accessible at http://www.fishpathogens.eu/noda as a freely available tool. We encourage researchers to register and add as many reports as possible as public available resources, although it is also possible to restrict data to the depositor only, or to a selection of registered users.

Queries and feedback on the database are welcomed (info@fishpathogens.eu).

Conclusions

In the last decades, betanodaviruses have provoked severe and recurrent epidemics worldwide, affecting

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several commercial fish species such as grouper, *Epinephelus* spp., sea bass, *Dicentrarchus labrax*, (L.), turbot, *Scophthalmus maximus*, (L.) and Atlantic halibut, *Hippoglossus hippoglossus*, (L.). More recently, outbreaks in freshwater farms and in marine natural ecosystems have clearly demonstrated that these viruses are able to spread and colonize new hosts and environments. This potential is a serious threat both commercially and ecologically in the context of the parallel increase of aquaculture and an increasing fragility of the natural fish populations. In case of new epidemics, it is of particular interest to identify the origin of the virus in order to prevent further introductions, by integrated chain management for instance. In this perspective, the new database should have an increasing role in tracking and tracing, among all the inventoried isolates, to find those exhibiting higher genetic similarities with the ones related to new disease outbreaks. For betanodaviruses, progressive accumulation of data in the database should demonstrate the distribution of the major genotypes, with special focus on the reassortants for which poor biological data are available. This emphasizes the need of collating genetic information on both RNA1 and RNA2 with epidemiological and biological data (e.g. virulence, water temperature) to investigate the ecological features and diffusive dynamics of betanodaviruses. In conclusion, the present database is a useful tool for all laboratory professionals working on VNN, to share data to better understand VNN and its spreading mechanisms, including molecular traits of betanodaviruses.

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Chapter 5

First generic one step real-time Taqman RT-PCR targeting the RNA1 of betanodaviruses

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First generic one step real-time Taqman RT-PCR targeting the RNA1 of betanodaviruses

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The detection of betanodavirus genomic components is a major issue for diagnostics and control of viral nervous necrosis (VNN), a devastating disease affecting fish worldwide. Despite a number of published molecular-based tests, most of them targeting the RNA2 molecule of the virus, diagnostics is still a challenge due to the high genetic diversity within this genus. In the present study, a new one-step real-time RT-PCR (rRT-PCR), targeting RNA1 of most genotypes of betanodaviruses, was proposed and validated. The test detected successfully various isolates of betanodavirus representatives of the four species RGNNV, SJNNV, TPNNV and BFNNV, either produced on cell culture or from clinical samples. It was specific as shown by the absence of signal on samples from healthy sea bass or from field samples of six other fish species without clinical signs of VNN. The assay detected reliably 50–100 copies of plasmids containing the targeted cloned RNA1 region, as well as an infectious dose of virus of $10^{2.5}$–$10^{2.85}$ $TCID_{50}$/ml. A set of samples was tested by two different laboratories, with similar results, demonstrating the robustness of the test. This is the first one step generic rRT-PCR method for betanodaviruses. It is simple to perform and may be used for first intention diagnostics as well as for confirmation in case of doubtful results obtained with other published tests targeting RNA2.

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1. Introduction

Viral nervous necrosis is a severe pathology affecting a large range of fish species produced through aquaculture or living in the wild (Kara et al., 2014; Munday et al., 2002; Vendramin et al., 2013). This disease has been found mostly in marine fish, although there has been an increasing number of reports of outbreaks in freshwater fish over the years (Athanassopoulou et al., 2003, 2004; Bigarré et al., 2009; Binesh, 2013; Bovo et al., 2011). The viruses responsible of this pathology belong to the genus *Betanodaviridae*. Their genome is composed of two RNA molecules, RNA1 and RNA2, of about 3.1 and 1.4 kb respectively. RNA1 encodes an RNA-dependent RNA polymerase (RdRp), as well as B2, an additional small protein translated from a subgenomic RNA (RNA3). RNA2 encodes the capsid (CP), which is the only structural protein of the virion.

At least four viral species are now recognized (www.ictvonline.org): red-spotted grouper nervous necrosis virus (RGNNV), Striped jack nervous necrosis virus (SJNNV), Barfin flounder nervous necrosis virus (BFNNV) and Tiger puffer nervous necrosis virus (TPNNV). An additional genotype, Turbot nervous necrosis virus (TNNV), has been defined and is currently awaiting classification as a genuine species (Johansen et al., 2004). Within a viral species, both genomic components are subjected to mutations (substitutions) and exhibit high variability despite relatively strong purifying selection on most codons (Panzarin et al., 2012). For some species, such as RGNNV for instance, sub-groups are distinguished and within these clusters viral populations are structured into quasi-species (Hick et al., 2013; Panzarin et al., 2012).

An additional factor of genetic variability is the possibility of reassortments within the genus. This mechanism has been described for RGNNV and SJNNV, for which isolates with RNA1 related to one particular species and RNA2 related to another species have been found in the Mediterranean (*i.e.* RGNNV/SJNNV and SJNNV/RGNNV) (Olveira et al., 2009; Panzarin et al., 2012; Toffolo et al., 2007). This emphasizes the importance of obtaining sequences from both RNA1 and RNA2 in order to correctly identify a given isolate.

Several factors certainly contribute to the genetic diversity and spread of betanodaviruses: the multiplicity of hosts and

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environments, the international trade of contaminated fish and the lack of standardized methods to select healthy carriers. As for clinical cases, their detection is increasingly performed by PCR, which provides rapid results, but is sometimes complicated by the difficulties of finding molecular probes to perfectly match the viral genome of an unknown sequence. Some PCR tests are well-adapted to one or two viral genotypes and have proved useful for detecting viral populations with known genetic profiles (Hick and Whittington, 2010; Hodneland et al., 2011; Nerland et al., 2007). A major break-through has been the development of a two-step real-time Taqman RT-PCR assay (rRT-PCR) that detects all RNA2 betanodaviruses in clinical specimens (Panzarin et al., 2010). However, a one-step generic method, also based on the use of a Taqman probe, would be useful both to increase the speed of the test and reduce the risk of contamination during the manipulation of genetic material. Targeting RNA1 would also be interesting in order to confirm doubtful results obtained with RNA2, for instance when viral loads reach the limits of detection or when rare mutations in the targeted regions affect the binding of the probes and consequently reduce the sensitivity of the test. In the present study, a one-step PCR test targeting the RNA1 of the vast majority of known viral genotypes is proposed and validated.

2. Materials and methods

2.1. Betanodavirus isolates

Globally, the betanodavirus genotypes covered four species: RGNNV, SJNNV, BFNNV, and TPNNV, including two types of reassortants between RGNNV and SJNNV. The origins of the viral samples tested were diverse (Table 1). Twenty-one isolates originating from the virus collections maintained at ANSES and IZSVe were multiplied in SSN1 cells as already described (Castric et al., 2001). Five isolates (W80, BB09, EE2, 484-2/I09, 389/I96) produced in cell culture and representing five very distinct genotypes (RGNNV, BFNNV, RGNNV/SJNNV, SJNNV and SJNNV/RGNNV respectively) were used to infect healthy sea bass in controlled conditions at ANSES or IZSVe. These animals were from farms free of nervous necrosis and controlled annually for the absence of virus by cell culture. The brain of these fishes were collected after a few days once the typical clinical symptoms (swirling) appeared. Besides the experimentally infected fish, 15 samples were obtained from clinical cases collected during disease outbreaks in farms or in the wild. Among all the positive samples, 13 were analyzed by two laboratories. In addition, the RNA from an isolate (W80) was produced, serially diluted and tested in both laboratories.

2.2. Primers and probe design

To select primers and a probe, RNA1 sequences from RGNNV, SJNNV and BFNNV were collated from GenBank and aligned with VNTI11 (Invitrogen). At the time this work was done, the TPNNV sequence (EU236148) was not yet available in GenBank. Therefore, a partial sequence of this virus was obtained by PCR amplifying a short region with the two primers selected below. The alignments were visually scrutinized for conserved blocks. GC-rich regions were not considered, in order to obtain an efficient amplification. Two primers were chosen in a conserved region at the 3′ end of RNA1. oPVP154 (5′ TCCAAGCCGGTCCTAGTCAA 3′) and oPVP155 (5′ CACGAACGTKCGCATCTCGT 3′) amplify a 168 or 171 nt-long sequence which includes a conserved target for a Taqman tqPVP16 probe (SIGMA) (Cy5-CGATCGATCAGCACCTSGTC-BHQ2) (Fig. 1). Both oPVP155 and tqPVP16 were degenerated in one position. The probe and primer oPVP155 were included within the RNA3 while oPVP154 shared only 7 nt with this subgenomic molecule.

2.3. RNA extraction, plasmids preparation and RT-PCR

Viral RNAs were extracted with a Nucleospin RNA virus kit or a Nucleospin RNAII kit (Macherey-Nagel) at ANSES and IZSVe respectively. The starting material was either 150 μl of cell culture supernatant or 150 μl of supernatant of organ pool (brain and eyes) ground up in cold PBS (10%, w/v) and centrifuged 3 min at 5000 × *g* in a microfuge. RNAs were resuspended in 50 μl of DEPC-treated water. The RNA extracted from the organs was quantified with a Nanodrop (Thermo Scientific) and diluted to 100 ng/μl for the RT-PCR assays.

The RT-PCR reactions were performed in 25 μl with 600 nM of each primer, 400 nM of probe, 5 μl of RNA and 1× of Quantitect Probe RT-PCR master mix (Qiagen). The following protocol was performed at ANSES and IZSVe with a CFX96 (Biorad) and a RotorGene 6000 (Corbett), respectively: 30 min at 50 °C, followed by 15 min at 95 °C, and 40 cycles of denaturation/extension for 15 s at 94 °C and 60 s at 60 °C.

Five reference plasmids were created by inserting the product of amplification of five distinct genotypes into the pCR4-TOPO TA-cloning vector (Invitrogen). These isolates were W80 (RGNNV), EE2 (RGNNV/SJNNV), Y197 (SJNNV/RGNNV), TPKag (TPNNV) and 484.2 (SJNNV). The plasmids were used at ANSES to estimate the sensitivity of the PCR phase, using the same RT-PCR protocol as for viral RNA.

2.4. Evaluation of the rRT-PCR assay

To evaluate the efficiency and the repeatability (intra- and inter-assays variabilities) of the rRT-PCR, standard curves were obtained starting with seven dilutions (10^{-1}–10^{-7}) of total RNA extracted from cell culture inoculated with a RGNNV strain (W80). The same PCR machine and the same batches of reactants were used. For the inter-assay variation, two operators from the same laboratory performed a total of four runs, each one at different days over 3 months. One operator performed two runs in triplicate and one run in duplicate (56 values obtained). The second operator performed a run in duplicate (14 values). A total of 70 values were obtained. Coefficients of variation (CV) were calculated using the ratio of the standard deviation to the mean (σ/μ) of the Ct values.

The robustness of the method was evaluated by varying by ±20% the concentrations of primers and probes for eight replicates of the same sample (RNA from isolate W80).

In a first approach, the sensitivity of the PCR step was evaluated by testing serial dilution series of various plasmids containing the targeted sequences from different genotypes (RGNNV, RGNNV/SJNNV, SJNNV/RGNNV, TPNNV, SJNNV). For each plasmid, 24 samples were ran (8 replicates × 3 runs) and the limit of the assay was defined when at least 23 of 24 identical samples were positive. In a second approach, the entire rRT-PCR method was evaluated by testing viruses produced in cell culture and titrated. Basically, Ct values of individual dilutions were plotted against the initial viral titer. With the same objectives, two different protocols were used by ANSES and IZSVe. At IZSVe, 10-fold serial dilutions of titrated cell culture supernatant infected with strain 283.2009 (RGNNV-type, GenBank JN189865) were prepared. The RNA was extracted in triplicate from each dilution, starting with an initial viral titer of $10^{7.50}$ $TCID_{50}$/ml. The rRT-PCR assay was performed in simplicate for each RNA extracted, according to the protocol reported above, using the Rotor-Gene 6000. At ANSES, strain W80 (RGNNV-type, GenBank AJ698107) was produced in cell culture ($10^{7.85}$ $TCID_{50}$/ml) and serially 10-fold diluted. Each viral dilution was extracted for RNA once and tested by rRT-PCR in triplicate under the conditions identical to those described above, using a CFX96 machine.

In order to assess analytical specificity, a large range of viral genotypes covering four betanodavirus species was tested by

Table 1
List of samples tested by real-time RT-PCR. For the betanodaviruses, the name of the host where the virus was first isolated, the geographical region and the year of isolation (cell line) or sampling (fish tissue) are indicated. The latin name of the host is indicated for its first mention in the table. The results of the real-time RT-PCR (Ct) obtained by ANSES and/or IZSVe are indicated in the two last columns. The second column indicates the laboratory which produced the samples exchanged between IZSVe and ANSES. Fish samples found negative were either sea bass from a virus-free farm or samples received for routine testing. Shaded: samples used for the reproducibility test (triplicated). RNA of W80 produced in cell culture has been tested at four dilutions (10^{-1}–10^{-4}).

Type of sample	Isolate	RNA1	RNA2	Host species of origin	Geographic origin	Year	Ct (ANSES)	Ct (IZSVe)
Viruses from cell culture	AA40	nd	RGNNV	Red drum (*Sciaenops ocellata*)	Mascarenes	2002	20.48	nt
	BB09	BFNNV	BFNNV	Sea bass (*Dicentrarchus labrax*)	France (Atl.)	2003	22.99	nt
	CC143	nd	SJNNV	Gilthead seabream (*Sparus aurata*)	Portugal	2004	16.76	nt
	DD10	RGNNV	RGNNV	Sea bass	Greece	2005	24.24	nt
	V82	RGNNV	RGNNV	Sea bass	Tunisia	1992	19.27	nt
	X90	nd	RGNNV	Sea bass	France (Med.)	1999	30.6	nt
	Y197	SJNNV	RGNNV	Red drum	France (Med.)	2000	23.62	nt
	Z271	nd	RGNNV	Sea bass	France	2001	24.64	nt
	DD20	RGNNV	SJNNV	Senegalese sole (*Solea senegalensis*)	Spain	2005	31.62	nt
	TPKAg93	TPNNV	TPNNV	*Takifugu rubripes*	Japan	1993	13.26	10.72[a]
	283.2009	RGNNV	RGNNV	Sea bass	Italy	2009	nt	16.19
	475/I98	RGNNV	RGNNV	Sea bass	Italy	1998	19.90	17.98
	512/I00	RGNNV	RGNNV	Sea bass	Italy	2000	16.50	14.04
	AH95NorA	BFNNV	BFNNV	*Hippoglossus hippoglossus*	Norway	1995	18.83	16.68
	BF93Hok	BFNNV	BFNNV	*Verasper moseri*	Japan	1993	16.35	13.60
	389/I96	SJNNV	RGNNV	Sea bass	Italy	1996	24.97	23.96
	367.2	RGNNV	SJNNV	Sea bass	Italy	2005	15.06	12.67
	484-2/I09	SJNNV	SJNNV	Senegalese sole	Spain	2009	16.83	14.46
	Y106	RGNNV	RGNNV	Barramundi (*Lates calcarifer*)	Tahiti	2000	22.56	21.24
	W80	RGNNV	RGNNV	Sea bass	France	1998	24.94	21.93
	W80 (10^{-1})						28.53	25.85
	W80 (10^{-2})						32.06	29.79
	W80 (10^{-3})						35.88	33.54
	W80 (10^{-4})						No Ct	35.52
Infected fish tissues	BB09[a]	BFNNV	BFNNV	Sea bass	France (Atl.)	2003	29.61	28.05
	EE2[a]	RGNNV	SJNNV	Gilthead seabream	Greece	2006	23.50	21.24
	W80[a]	RGNNV	RGNNV	Sea bass	France	1998	25.13	22.82
	389/I96[a]	SJNNV	RGNNV	Sea bass	Italy	1996	nt	25.66
	484-2/I09[a]	SJNNV	SJNNV	Senegalese sole	Spain	2009	nt	32.99
	07/211 FF139	RGNNV	RGNNV	Tilapia (*Oreochromis niloticus*)	Belgium	2007	22.46	nt
	09/126	RGNNV	SJNNV	Gilthead seabream	France (Med.)	2009	24.95	nt
	09/131F	RGNNV	RGNNV	Sea bass	Cyprus	2009	14.48	nt
	12/050 EM	RGNNV	RGNNV	Dusky grouper (*Epinephelus marginatus*)	Algeria	2011	20.72	nt
	12/062	RGNNV	SJNNV	Senegales sole	Greece	2012	14.23	nt
	12/105	RGNNV	RGNNV	Sea bass	Greece	2012	14.73	nt
	12/107	RGNNV	RGNNV	Dusky grouper	Algeria	2012	29.16	nt
	12/108	RGNNV	RGNNV	Goldblotch grouper (*Epinephelus costae*)	Algeria	2012	20.11	nt
	12/109	RGNNV	RGNNV	Dusky grouper	Algeria	2012	17.63	nt
	12/110	RGNNV	RGNNV	Dusky grouper	Algeria	2012	19.36	nt
	12/114	RGNNV	RGNNV	Dusky grouper	Algeria	2012	21.97	nt
	12/115	RGNNV	RGNNV	Dusky grouper	Algeria	2012	23.01	nt
	12/116	RGNNV	RGNNV	Sea bass	Algeria	2012	32.58	nt
	12/128	RGNNV	RGNNV	Dusky grouper	Algeria	2012	23.04	nt
	13/025	RGNNV	RGNNV	Dusky grouper	Algeria	2013	23.5	nt
Fish samples found negative	Healthy sea bass			Sea bass	France	2011	No Ct	No Ct
	10/008			Sea bass	Spain	2010	No Ct	nt
	10/061			Red drum	Mascarenes	2010	No Ct	nt
	10/065			Sea bass	Greece	2010	No Ct	nt
	11/123 1			Tilapia	Israel	2011	No Ct	nt
	12/111			Dusky grouper	Algeria	2012	No Ct	nt
	12/112			Goldblotch grouper	Algeria	2012	No Ct	nt
	12/113			Goldblotch grouper	Algeria	2012	No Ct	nt
	12/129			*Chelon labrosus*	Algeria	2012	No Ct	nt
	13/026			*Dentex dentex*	Algeria	2012	No Ct	nt
	13/027			Sea bass	France	2013	No Ct	nt
	13/028			Sea bass	France	2013	No Ct	nt
	13/029			Sea bass	France	2013	No Ct	nt
	13/036			Sea bass	France	2013	No Ct	nt
	13/037			Sea bass	France	2013	No Ct	nt
	13/038			Sea bass	France	2013	No Ct	nt
Other viruses (cell culture)								
	VHSV 07/71			Rainbow trout (*Oncorhynchus mykiss*)	France	1971	No Ct	nt
	VHSV 860/I94			Turbot (*Scophthalmus maximus*)	Scotland	1994	No Ct	No Ct
	IHNV 11/003			Rainbow trout	France	2011	No Ct	nt
	IPNV EE152			Rainbow trout	France	2006	No Ct	nt

Med., from the Mediteranean coast; Atl., from the Atlantic coast; Nd, not determined; Nt, not tested.
[a] samples from sea bass experimentally infected; a value obtained in simplicate.

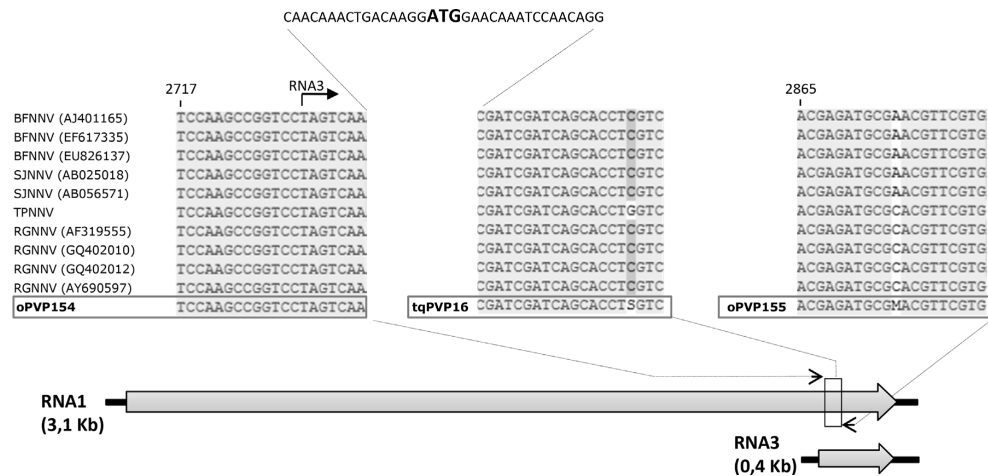

Fig. 1. Position of the new primers and probe within the RNA1 molecule. The oligonucleotides were aligned with the full-length RNA1 sequences available in Genbank or sequenced in this study (TPNNV). As a reference, the position of the primers and probe on a BFNNV genome (AJ401165) is indicated above the alignment, as well as the start of the subgenomic RNA3 and the AJ401165-specific sequence flanking the start codon of the B2 protein (bold) (Sommerset and Nerland, 2004).

rRT-PCR. As negative controls, RNA from healthy fish as well as viruses from two other families (*Rhabdoviridae* and *Birnaviridae*) were used, namely viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). Other fish samples of unknown health status and various origins were analyzed with the new RT-PCR test.

Among all the samples, a selected set from various origins was tested both by ANSES and IZSVe (in triplicate) with different batches of the same reactant references, but using different PCR machines. The data obtained was also used to evaluate the robustness of the method. In only one case (TPNNV), the tests were made in duplicate and simplicate, in each laboratory respectively.

3. Results

3.1. rRT-PCR efficiency, intra- and inter-assay variation

Ct values were obtained starting with 10-fold dilutions of total RNA extracted from cell culture inoculated with an RGNNV strain. The linear regression of the curves was superior to 0.99 ($R^2 = 0.995$) and the efficiency was 93.9%. The same data were used to evaluate intra-assay variability. The CV of Ct values varied between 0.05 and 1.1% indicating a high level of intra-assay repeatability (Table 2a). In order to evaluate the inter-assay variability, the data from the intra-assay test were compiled with those obtained from three additional assays performed the same week or 3 months later, by the first operator or a second one. Out of a total of 70 values extracted from the four assays, the CV varied between 0.43 and 1.78% demonstrating that the considered variables had little effect on the signal (Table 2b). In light of this data, the assay proved to be repeatable.

3.2. Robustness

The robustness of the assay was tested by varying the concentrations of the primers and probes in the reaction (Fig. 2). At the recommended concentration and at −20 or +20%, the Ct values were 28.63, 28.58 and 28.6 with CVs of 0.31, 0.6 and 0.19% respectively. The test was therefore considered robust when subjected to slight modifications of the concentrations of the primers and probe.

3.3. Analytical sensitivity

Serial dilutions of a plasmid containing the targeted sequence were tested with the rRT-PCR assay. Preliminary tests indicated that the limit of detection was between 20 and 100 copies of plasmid. Therefore, three quantities (20, 50 and 100 copies) of five plasmids containing each a genomic portions of a particular genotype were further tested, in three independent runs, each quantity represented by 8 identical samples (total 24 samples/plasmid). In total, 360 runs were performed and the positive samples were

Table 2
Repeatability of the RT-qPCR assay assessed based on the intra- and inter-assay variations. The Ct mean, standard deviation (SD) and coefficient of variation (CV) were determined by testing serial dilutions of RNA extracted from cell supernatant and diluted from 10^{-1} to 10^{-7}.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
(a)							
1 session							
Ct Mean ±SD	14.91 ± 0.05	17.82 ± 0.4	21.37 ± 0.03	24.60 ± 0.01	27.91 ± 0.04	31.44 ± 0.09	36.26 ± 0.4
CV (%)	0.30	0.21	0.14	0.05	0.16	0.28	1.10
(b)							
4 sessions							
Ct Mean ±SD	14.93 ± 0.08	17.98 ± 0.32	21.63 ± 0.23	24.79 ± 0.14	28.36 ± 0.42	31.62 ± 0.14	36.80 ± 0.48
CV (%)	0.54	1.78	1.09	0.58	1.47	0.43	1.31

(a) Intra-assay precision: data from one assay performed with triplicates.
(b) Inter-assay precision: data from four assays made at different days and by two operators.

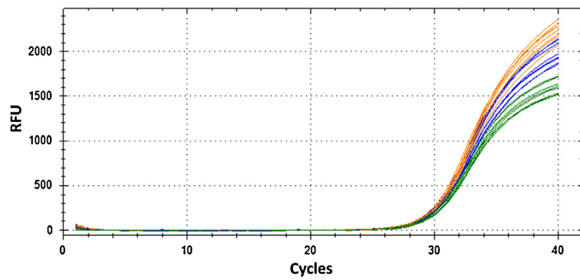

Fig. 2. Robustness of the RT-qPCR test. Three concentrations of primers and probes were tested with the same amount of RNA extracted from infected cell supernatant. Orange: +20%; blue: recommended; green: −20% concentration of primers and probe. RFU, relative fluorescence units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

counted. With 100 copies, all the samples of the five plasmids were found positive (mean Ct of 35.87 ± 0.47). With 50 copies, all samples from three plasmids were fully positive (24/24) while samples from two others reached only 92% of positive (22/24). There was therefore a slight difference between plasmids containing different sequences. As expected, the proportions of positive were even lower (58 to 83%) with 20 copies, whatever the plasmid. Finally, the limit of detection was considered to be 100 copies of dsDNA carrying a betanodavirus sequence, with a 95% confidence level.

The sensitivity of the assay was further evaluated by testing two titrated isolates (W80 and 283.2009) produced in cell culture. The limit of detection (LoD) of the rRT-PCR method was arbitrarily defined as the last dilution for which at least 2 out of 3 replicates tested positive. At IZSVe, the assay proved capable of detecting betanodavirus RNA1 of 283.2009 down to a dilution of 10^{-5}, corresponding to $10^{2.50}$ $TCID_{50}$/ml. At ANSES, viral RNA1 of W80 was detected down to 10^{-5}, corresponding to $10^{2.85}$ $TCID_{50}$/ml.

3.4. Analytical specificity

A range of isolates representing four different betanodaviruses, RGNNV, SJNNV, BFNNV, TPNNV and two reassortants was tested with the proposed rRT-PCR. Most isolates were produced in cell culture ($n = 21$) or derived from clinical cases ($n = 15$) from six different host species of various geographical origins. Five additional samples were extracted from sea bass experimentally infected with known isolates of RGNNV, RGNNV/SJNNV, SJNNV, SJNNV/RGNNV or BFNNV. All these samples gave Ct values varying between 10.72 and 32.58 (Table 1). These results indicated that the assay efficiently detected strains belonging to at least four species, as expected.

As additional controls of specificity, three other RNA viral species, VHSV (two strains), IHNV and IPNV, were tested. No signal was produced for these four viruses. A series of negative controls operated with RNA extracted from healthy control fish (sea bass) or from fish with no clinical signs were also negative.

During the testing of the collection of isolates with probe tqPVP16, sample Y106 produced an unusual fluorescence signal (Fig. 3). Although the Ct varied from 17.5 to 35.5 (dilutions of 10^{-2}–10^{-7}), the amplification plot displayed a shape different from that expected, with a final fluorescence approximately twice as low as the other Ct samples in the same range (isolate V82 for instance). To explain this observation, an imperfect binding of the probe to the viral RNA was hypothesized. The sequence of the amplified region of Y106 was therefore obtained and confirmed the presence of a mismatch between the target sequence and the probe, at the exact position of the degeneration (T instead of S).

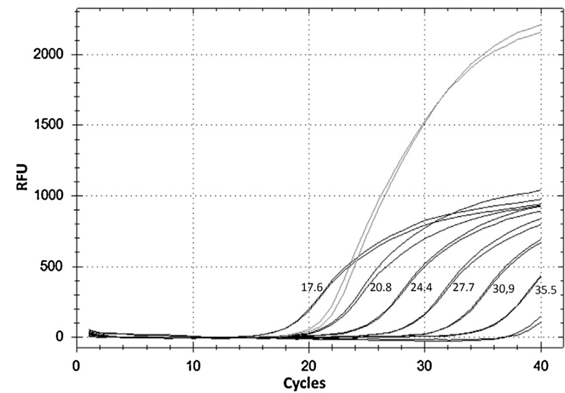

Fig. 3. Results of RT-PCR with isolate Y106. Black curves are 10-fold serial dilutions (10^{-2}–10^{-8} in duplicates) of RNA. Mean Ct is indicated near the curves. No Ct was obtained for dilution 10^{-8}. In gray, isolate V82 with Ct = 20.7.

3.5. Reproducibility

To evaluate the reproducibility of the method, identical samples were aliquoted and tested by two laboratories. In total, 18 samples (16 positives and two negatives) were submitted to rRT-PCR. For betanodavirus-containing samples, the signals for IZSVe and ANSES were equivalent, though consistently slightly higher for IZSVe (Fig. 2). This minor difference was attributed to the PCR apparatus since the reactants were identical. Consequently, in one single case, one sample highly diluted (W80) gave a positive, though very weak, signal at IZSVe, and was negative at ANSES. The negative controls exhibited no signal in both labs. Therefore, the results were fully consistent between the two laboratories and the method was considered reproducible.

4. Discussion

Betanodaviruses have a strong impact on marine aquaculture in many regions worldwide. In particular, the Mediterranean has been hit by many outbreaks in recent years. Although no estimations are available, a very strong impact on sea bass farms in this region is widely recognized. The situation has become serious for some wild fish populations, with repeated outbreaks affecting wild grouper in recent years (Kara et al., 2014; Vendramin et al., 2013). Obvious explanations for these high losses worldwide include the intensification of farming of susceptible species, the intense fish trade which contributes to the dissemination of viruses, and a lack of active surveillance programs and control measures.

The need to strengthen efforts to control this disease has now become urgent. The availability of efficient standardized diagnostic tests is a prerequisite to a global strategy. Previous studies have demonstrated the superiority of real-time PCR over other diagnostic methods, such as cell culture or nested-PCR (Dalla Valle et al., 2005; Hick et al., 2011; Panzarin et al., 2010). However, the high genetic diversity of the betanodaviruses is a challenge for the selection of primers capable of binding with high affinity to all the possible variants. Methods described in other studies have deliberately targeted selected viral genotypes, for instance those endemic in a particular geographical region (Hodneland et al., 2011; Korsnes et al., 2005). Only one study proposed molecular probes designed to recognize the RNA2 of all viral species with high efficiency (Panzarin et al., 2010). However, development of an independent assay targeting RNA1 and used either as a first line choice or alternatively as a confirmatory method for those samples yielding high Ct or doubtful results with RNA2 is needed.

In order to set up a one-step generic RT-PCR assay, two primers and one probe were chosen in a highly conserved region, situated in the 3′ part of RNA1. Within these three oligonucleotides, only two positions were degenerated. The RNA1 genomic component had rarely been used for molecular diagnostics despite its potential for first line diagnosis (Dalla Valle et al., 2005). Some authors tried to set up a Taqman assay targeting RNA1, which proved to be more sensitive than the two assays targeting RNA2 (Hick and Whittington, 2010). However, this RNA1 Taqman assay was not suitable since it detected only RGNNV and produced some few false positives. Conversely, other authors have found that RdRp RNA was 2–10 times less abundant than CP RNA (RNA2), suggesting that targeting RNA2 would lead to more sensitive diagnostics (Dalla Valle et al., 2005). Unfortunately, it was impossible to find within RNA2 three highly conserved short sequences adapted to a Taqman assay, unless by introducing a number of degenerations. An additional advantage of targeting RNA1 is that it could be used as a confirmation test in situations where a first line diagnostic test targeting RNA2 produces a doubtful result.

It was not determined whether RNA3 was detected by the new Taqman test. Indeed, primer oPVP155 and the probe are included in its sequence. However, only 7 nt of the 3′ part of primer oPVP154 binds to the 5′ part of RNA3, which certainly strongly reduces, but may not completely abolish, priming events during the PCR (Fig. 1). Considering that RNA3 is very abundant comparatively to RNA1 during the first 24 h of replication of BFNNV in cell culture (Sommerset and Nerland, 2004), it is conceivable that a percentage of RNA3 is possibly detected with the new test, contributing to the positive signal. More knowledge is needed concerning the kinetics of RNA3 in fish and its use as a target for diagnostics.

When used in conventional PCR, the oPVP154-155 primer set proved to be useful alone to detect RGNNV isolates during an epidemiological study in sea bass and sea bream in Tunisian farms (Haddad-Boubaker et al., 2013). A Taqman probe was therefore added to the method to increase specificity in an attempt to extend the method to the widest possible panel of hosts.

The assay reliably detected 50–100 copies of plasmids containing a fragment of viral genome. In comparison, 10 copies of a recombinant plasmid were detected by other authors targeting a different region of RNA1, although the analytical sensitivity was not extensively verified (Dalla Valle et al., 2005). In another study, a level of 3×10^2 copies/μl was reached working with RNA transcribed *in vitro* (Panzarin et al., 2010).

In the present work, viral loads were detected on six different host species, dusky and goldblotch groupers, sole, sea bass, sea bream and tilapia. However, more than 50 hosts have now been described as carriers of betanodaviruses (Vendramin et al., 2013). Additional tests on other hosts will be needed to evaluate the extent of applicability of the method.

The new RT-PCR assay was able to detect genotypes representative of the four recognized species of betanodaviruses, as well as five reassortants between RGNNV and SJNNV. Among the latter five isolates, two were detected by rRT-PCR using virus produced in cell culture and three were identified directly from experimentally infected fish. Only TNNV was not available and not tested. Therefore, the assay had the potential to detect a large range of genotypes.

Strikingly, one isolate (Y106) exhibited an atypical signal, with a low Ct value and a pronounced reduction in final fluorescence. This type of signal could be the consequence of a mismatch between the probe and the targeted viral genome. It must be mentioned that, before designing the tqPVP16 probe, preliminary assays were performed with a first probe quasi-identical to tqPVP16, excepted that it was not degenerated (S → C). For all the betanodavirus isolates tested at this time, this first probe produced strong signals for most isolates, with the exception of TPNNV for which no signal was produced, either by ANSES, or by IZSVe (data not shown). Surprisingly, the sequencing of this region revealed a single nucleotide difference between the viral genome and the probe. Therefore, a new probe (tqPVP16) was synthesized by degenerating one position (C → S). A high signal was recovered for TPNNV demonstrating the strong impact of a specific nucleotide mismatch at that position.

It is known for Taqman probes that a single mismatch in the binding region may have a pronounced effect on both target quantification and endpoint fluorescence, while affecting efficiency only slightly (Süβ et al., 2009). In the case of Y106, the detection was still positive despite the mismatch, possibly because of the moderate negative effect of an S–A mispair on the hybrid probe-target. S stands for C or G and leads to C–A or G–A mispairs for this isolate. In the literature, it was described that G–A mispairs in the primer-template induced a severe impact on the Ct while C–A mispairs had less effect (Ke and Wartell, 1993; Stadhouders et al., 2010). In comparison, for TPNNV tested with the preliminary probe tqPVP14, the effect of the mispair (C–C) was the complete absence of any signal, which might be explained by the known highly destabilizing effect of this particular mispair. While mutations at this site had been found for TPNNV and Y106 in the past, no changes were observed among the more than 70 sequences found in GenBank at that time, including representatives of RGNNV, SJNNV and BFNNV. Thus, although these changes seem rare, it would be worthwhile in the future to sequence this region in all new isolates in order to evaluate the frequency of changes in the viral populations.

In conclusion, the new test is the first generic RNA1 one-step Taqman-based rRT-PCR. It is rapid, sensitive, specific and has a potential for being used routinely in different geographic regions and host species with a very high chance of detecting any isolate. An additional advantage of the targeted region is its potential, after sequencing, for providing a rough genotyping in order to identify the viral species (Haddad-Boubaker et al., 2013). In addition, the region targeted by the Taqman probe is subjected to rare variations. This implicates that the new assay, although applicable to a very wide range of genotypes, may exhibit certain limitations depending on the frequency of mutations in the viral populations. This emphasizes the importance in the future of investing in a continual sequencing effort for the characterization of viral diversity on the widest scale, not only to shed light on the circulation of genotypes between regions and hosts, but also to identify variants that might escape diagnosis. Furthermore, it is important, when possible, to test doubtful samples using two methods in order to avoid false negatives, for instance cell culture and PCR, or ideally two PCRs targeting two different regions of the viral genome. While the tool presented here will contribute to the surveillance and control of betanodaviruses, epidemiological studies will still be needed in order to continuously adapt the molecular diagnosis to evolving viral species.

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Chapter 6

Molecular basis for antigenic diversity of genus betanodavirus

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Paper submitted

Molecular basis for antigenic diversity of genus *Betanodavirus*

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Abstract

Betanodaviruses are the causative agents of viral nervous necrosis (VNN), a devastating disease for the Mediterranean mariculture. Four different betanodavirus species are recognized, Striped jack-, Redspotted grouper-, Tiger puffer-, and Barfin flounder nervous necrosis virus (SJNNV, RGNNV, TPNNV and BFNNV), but there is little knowledge on fish nodaviruses antigenic properties. In the present study, serum neutralization assays were performed using rabbit polyclonal antisera against eight betanodaviruses that cover a wide species-, temporal-, spatial- and genetic range. The results indicate that the SJNNV and RGNNV are antigenically distinct, constituting serotypes A and C, respectively. The TPNNV and BFNNV, the latter representing cold-water betanodaviruses, are antigenically related and cluster within serotype B. The reassortant viruses RGNNV/SJNNV and SJNNV/RGNNV group within serotypes A and C, respectively, indicating that the coat protein encoded by RNA2 acts as major immunoreactivity determinant. Immunostaining of *in vitro* expressed wild type and chimeric capsid proteins between the RGNNV and the SJNNV genotypes indicated that the C-terminal part of the capsid protein is involved in the antigenic diversity. The amino acid (aa) residues determining RGNNV and SJNNV antigenic diversity were mapped to aa residues 217-256 and aa 257-341, respectively. Neutralization of reverse genetics derived chimeric viruses indicated that these areas determine the neutralizing epitopes. The data obtained are crucial for the development of targeted serological tests for the diagnosis of VNN, and informative for development of cross-protective vaccines against various betanodavirus genotypes.

Introduction

Decreasing wild stocks combined with increasing consumer demand for fish have contributed to the rapid expansion of the aquaculture production in the last decades, with a global yield of approximately 47 million tons in 2013 [1]. However, in industrialized farming the fish are kept at high densities and thus vulnerable to various infectious diseases, which represent a major threat for the sustainability of aquaculture production [2]. Viral nervous necrosis (VNN) (synonymous viral encephalopathy and retinopathy - VER) is an important infectious disease of farmed fish, and is caused by betanodaviruses. VNN represents a main bottleneck for the farming of marine finfish, and causes disease outbreaks in species such as European sea bass (*Dicentrarchus labrax*), gilthead sea bream

(*Sparus aurata*), Senegalensis sole (*Solea senegalensis*), barramundi (*Lates calcarifer*) and groupers (*Epinephelus spp.*) [3-14]. Furthermore, VNN also impedes the introduction of new marine fish species as alternatives for farming [15]. Fish nodaviruses have global distribution and can infect a large number of different fish species [16]. They have a bi-segmented, single-stranded, positive sense RNA genome. The genomic segment RNA1 encodes the viral polymerase, and gives rise to the subgenomic transcript RNA3 that encodes proteins B1 and B2. The RNA2 segment encodes the coat protein (CP) [17-20]. The structure of recombinant virus-like particles (VLPs) of a nodavirus from *Epinephelus malabaricus*, as observed by electron cryomicroscopy, suggests that the portion between amino acids (aa) 217 and 308 of the coat protein putatively forms a protrusion in the outer part of the capsid [21]. In a more recent study, the crystal structure of a grouper VLP was resolved, and three major domains were identified: the N-terminal arm (aa 34-51) and the shell domain (S-domain) (52-213) connected through the linker region (aa 214-220) to the protrusion domain (P-domain) (221-338) [22]. The P-domain harbors the putative host-specificity determinants of betanodaviruses [23] and the encoding nucleotide sequence includes the T4 variable region that can be utilized to classify fish nodaviruses into four distinct genotypes, namely RGNNV, SJNNV, TPNNV and BFNNV [24]. The BFNNV group also contains the psychrophilic betanodavirus strains of Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) [25,26]. Interestingly, the genetic diversity among fish nodaviruses is further increased by reassortment of genomic segments, as observed between the RGNNV and the SJNNV genotypes, resulting in the reassortant strains RGNNV/SJNNV and SJNNV/RGNNV [11,27,28]. Although diverse fish nodavirus genotypes possess a number of common antigenic determinants [29,30], the genetic variety among the four betanodavirus genogroups reflects the differences in their immunoreactivity. By means of indirect fluorescent antibody tests (IFAT) and serum neutralization assays (SN), Mori et al. classified fish nodaviruses into three different serogroups. Serotype A consists of SJNNV strains, serotype B correspond to the TPNNV genotype, and serotype C comprises the RGNNV and the BFNNV species [29]. However, this classification is mainly based on viral isolates from Asia and neglects cold-water strains as well as reassortants.

In order to provide an overview of the immunological properties of betanodaviruses, the present study aimed to cover the knowledge gap of the serological reactivity of

betanodaviruses from the Mediterranean, cold-water strains and reassortant viruses. Furthermore, to better understand the molecular traits responsible for betanodavirus immunoreactivity, the immunostaining of wild-type and chimeric coat proteins harboring amino acid residues of the RGNNV and the SJNNV viruses was performed, and wild-type and chimeric betanodaviruses synthetized through the reverse genetics (RG) were serologically characterized.

Materials and methods

Cell lines

E-11 fibroblasts, cloned from the SSN-1 cell line (striped snakehead, *Ophicephalus striatus*) [31] were grown at 25°C in L-15 medium (Leibovitz) (Sigma-Aldrich) containing 10% FCS, L-Glutamine (2mM) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B) [32].

The epithelioid cell line (EPC) from epithelial papilloma of common carp (*Cyprinus carpio*) [33] was maintained at 20 °C in L-15 medium (Leibovitz) (Life Technologies) supplemented with 10% FCS and gentamycin-sulfate (50 µg/mL).

Betanodaviral strains

Eight betanodaviruses were used to produce rabbit immune antisera: 283.2009 (RGNNV), 484.2.2009 (SJNNV), 367.2.2005 (RGNNV/SJNNV), 389/I96 (SJNNV/RGNNV), TPKag93 (TPNNV), JFIwa98 (BFNNV), SK-07 1324 (BFNNV) and Ah95NorA (BFNNV) (S1 Table). All but strains TPKag93 and JFIwa98 originate from Europe. Strain 484.2.2009 was provided by Dr. Francesc Padrós (Universitat Autònoma de Barcelona) and isolates SK-07 1324 and Ah95NorA were supplied by Dr. Hilde Sindre (Norwegian Veterinary Institute). Additional 11 field viruses isolated from diseased fish (*n*=8 RGNNV and *n*=3 RGNNV/SJNNV) (S1 Table) were serologically characterized blindly to evaluate the capability of the SN tests to serotype correctly. Viruses were selected to cover a wide variability in terms of host species, year of isolation, geographic origin and genetic features. The RNA2 sequences of the strains used in this work were retrieved from GenBank apart from SK-07 1324, Ah95NorA and E.marginatus/I/35-1/Dec13, which were obtained in the present study according to Panzarin et al. [34]. Pairwise nucleotide distances estimated with MEGA5 [35] and the phylogenetic tree describing the genetic

relationships existing between all 19 viral strains are given in supplementary material (S2 Table and S2 Figure).
The viruses were propagated in E-11 cells and after completion of cytopathic effect (CPE) media were collected, clarified at 4400 × *g* (Beckman centrifuge JA-21, rotor JA-10) for 60 min at 4°C and titrated. Viral titres, expressed as $TCID_{50}$/ml, were calculated according to the Spearman-Kärber formula [36].
The viral antigens for rabbit immunization were purified by precipitation by adding 8% w/v poly-ethylene glycol (BioUltra 8 000, Sigma-Aldrich) and 2.2% w/v NaCl and incubated at +4°C overnight with gentle shacking. Samples were then centrifuged at 4400 × *g* for 120 minutes at 4°C, the pellets were dissolved 1:100 in TEN buffer (Tris-HCl 40 mM; EDTA 1 mM; NaCl 150 mM, pH 7.6), and sonicated 5×30 seconds on ice. Viral suspension was layered on a caesium chloride continuous gradient (Sigma-Aldrich) (27-40% w/v) and centrifuged for 18 hours, 110000 × *g*, at +4°C (Beckman centrifuge L-100K, rotor SW41Ti). Virus bands were collected with a syringe and then precipitated by centrifugation at +4°C for 4 hours at 110000 × *g*. Pellets were dissolved overnight at +4°C in 1 to 3 ml of TEN buffer. Purified viruses were titrated in E-11 cells and final viral titres were determined as described above.

Rabbit antisera production

Antisera were obtained by immunizing six month-old New Zealand rabbits with subcutaneously injection of 0.3 ml of purified virus inactivated with 1% of formalin and emulsified with 0.7 ml of adjuvant Montanide ISA763VG (Seppic). This treatment was followed by 5 to 6 administrations of 0.5 ml live purified virus (without adjuvant) in the lateral ear vein. Inoculations were made at time intervals of 3 weeks. Local anaesthetic (lidocaine) was applied 20 minutes before each injection. Seroconversion was checked after the fifth intravenous injection, and when satisfactory titres were achieved (i.e. serum neutralization titres >1:1280/2560), the rabbits were bled to death after deep anaesthesia (pre-anaesthesia with 2 mg/kg acepromazine; anaesthesia with 7 mg/kg xylazine and 50 mg/kg ketamine). The serum was harvested and clarified by centrifugation. Rabbit hyperimmune sera were heat inactivated at 56°C for 30 minutes, aliquoted and stored at -20°C until use. The immunization procedure was approved by the Internal Ethic Commission (decision n° 3/2013 of the 13th May 2013) and authorized by the Italian

Ministry of Health (IZSVe protocol n° 0006230/2013 of the 19th June 2013) according to the National and the EU legislation.

Neutralization assays

To describe the serological relationship among betanodaviruses with diverse genomes, cross-SN tests were carried out in four independent replicates by testing each serum against each of the eight viruses used for immunization. Two-fold serial dilutions of serum (from 1:20 to 1:40960) were prepared in 96 well plates (Corning) with L-15 medium (Leibovitz) (Sigma-Aldrich) without FBS. Four parallel wells were used for each serum dilution. Diluted sera were incubated with 100 $TCID_{50}$/25μl of viral suspension. After an overnight incubation at +4°C, the virus-serum mixture was inoculated onto confluent E-11 monolayers and incubated for 10 days at 25°C (for the SJNNV, RGNNV, RGNNV/SJNNV and SJNNV/RGNNV genotypes) or at 20°C (for the TPNNV and BFNNV genotypes). Every 3 days cells were scrutinized for CPE appearance. The neutralizing titres were determined as the reciprocal of the highest dilution of serum capable to neutralize the virus in at least two out of four wells. Neutralizing titres were transformed into Log2 values. The geometric mean titre (GMT) was calculated among the four replicates and the value obtained was converted again into neutralizing titre. The Archetti and Horsfall formula was applied to estimate the 1/r index used to describe the serological relationships among different betanodavirus isolates [37]. A 1/r value of 10 or higher indicates that two viruses belong to distinct serological groups [38].

For blind evaluation of the SN assay, further 11 field strains were subjected to serological characterization by testing in single the whole panel of rabbit hyperimmune antisera following the procedure described above.

Statistical analysis

The Spearman coefficient (r_s), estimated on the Log2 neutralization titres, was used to evaluate the presence of serological correlation between two different viruses [39]. Spearman coefficient is a non-parametricrank statistic for measuring the strength of a monotonicrelationship between paired data, without making any assumption about the frequency distribution of the variables. The correlation coefficient ranges between -1 and 1. Absolute values of r_s are interpreted as follows: 0.00-0.19 "very weak" correlation; 0.20-

0.39 “weak” correlation; 0.40-0.59 “moderate” correlation; 0.60-0.79 “strong” correlation; 0.80-1.0 “very strong” correlation. Correlation coefficients with p-value < 0.05 were considered significant.

Furthermore, in order to identify groups of viruses showing analogous immunoreactivity profiles, the principal component analysis (PCA), based on the Log2 values of neutralizing titres, was also applied [40]. This analysis is a variable reduction technique that provides a new set of uncorrelated and ordered variables (i.e. principal components, PCs) which are a linear combination of optimally-weighted observed variables (i.e. viruses). The estimation of PCs is obtained by the eigenvalue decomposition of the variance/covariance matrix. The number of PCs were selected according to the Kaiser criterion (eigenvalue > 1) [41], the scree plot of the eigenvalues versus the number of the components, the proportion of variance for each component, the cumulative proportion of variance explained and the interpretability of the principal components.

The statistical analyses were carried out with the software SAS version 9.3 (SAS Institute, Cary, N.C.).

Plasmids preparation

Total RNA was purified from 100 µl of clarified supernatants infected with strains 283.2009 (RGNNV) and 484.2.2009 (SJNNV) using the NucleoSpin RNA II (Macherey-Nagel GmbH & Co.). cDNA was synthetized with the SuperScript® III Reverse Transcriptase (Life Technologies) following the manufacturer’s instructions and by adding 10% DMSO to the reaction mix. Viral cDNA was amplified using specific primer sets (S3 Table) and the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies) according to the producer’s recommendations. The pcDNA™3.1$^{(+)}$ Mammalian Expression Vector (Life Technologies) was used to express wild-type and chimeric betanodavirus coat proteins in EPC cells under the control of cytomegalovirus (CMV) promoter. Chimeric open reading frame (ORF) sequences were synthetized by exchanging parts of the RNA2 segment between the RGNNV (283.2009) and the SJNNV (484.2.2009) strains (Fig 1A).

For the generation of reverse genetics viruses, the full length wild-type RNA1 and RNA2 sequences of isolates 283.2009 and 484.2.2009, as well as chimeric RNA2 genes engineered as above, were ligated into pI18 plasmid (kindly provided by Dr. Nigel Temperton) (Fig 2B). Cloning was carried out using the In-Fusion® HD Cloning Kit

(Takara Bio Inc., Ōtsu, Japan) and the XL10-Gold® Ultracompetent Cells (Agilent Technologies) following the manufacturers' instructions. The sequences of the DNA inserts were verified by sequencing.

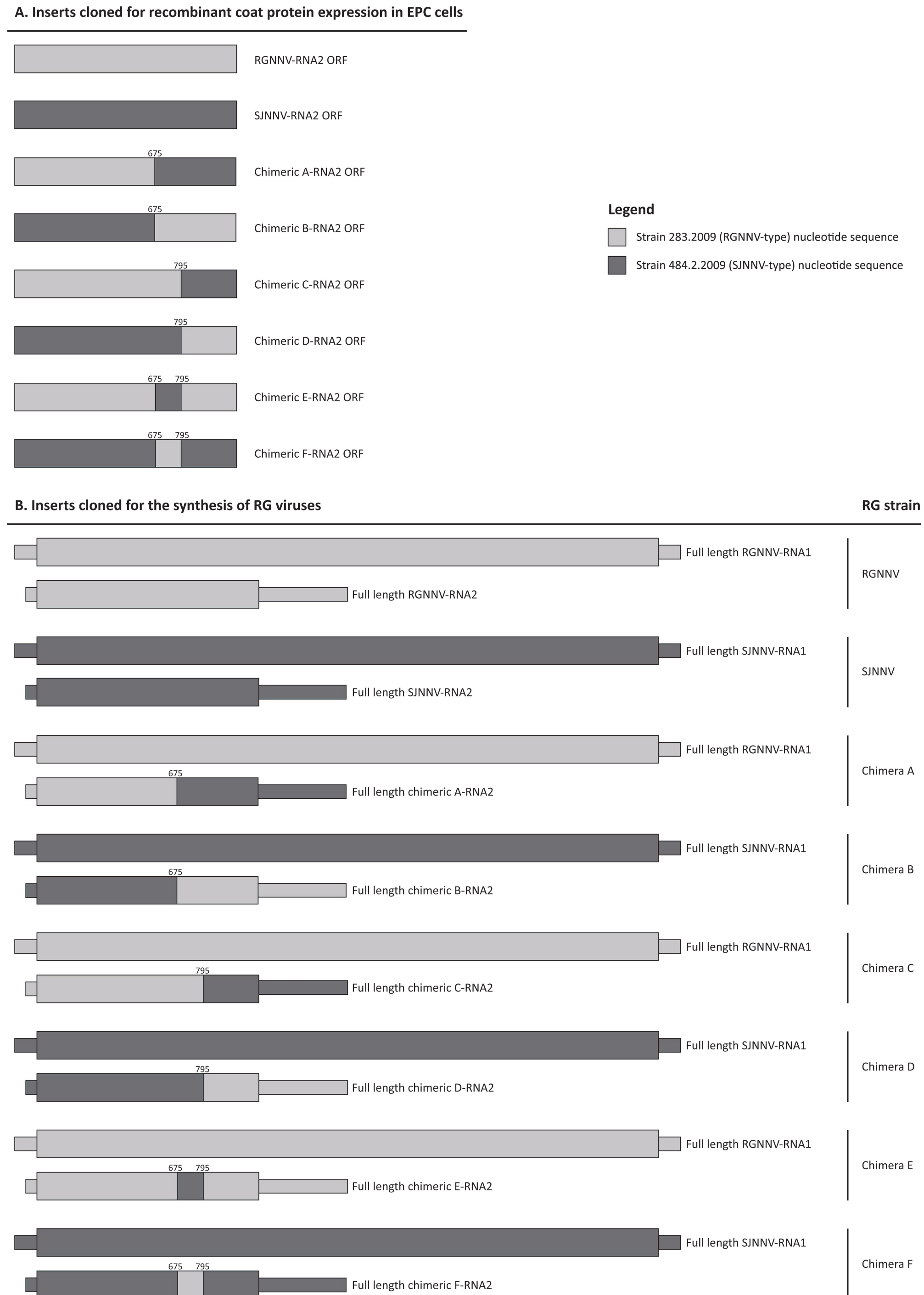
Recombinant capsid proteins expression and immunostaining

EPC cells were transfected with 2 μg of pcDNA™3.1$^{(+)}$ plasmids containing wild-type and chimeric RNA2 ORF sequences (Fig 1A) using the Ingenio® Electroporation Solution (Mirus) and the Amaxa Nucleofector™ Device (Lonza). After 24 hours incubation at 20°C, transfected cells were treated with 80% aceton. Fixated monolayers were then blocked with 10% foetal bovine serum (FBS) and subsequently immunostained for 1 hour at room temperature with rabbit polyclonal antibodies raised against betanodavirus strains 283.2009 (anti-RGNNV, diluted 1:20000) and 484.2.2009 (anti-SJNNV, diluted 1:3000). To avoid unspecific reactions, sera had previously been conditioned against EPC cells. Subsequently, washed cells were incubated for 45 min at room temperature with Alexa Fluor® 488 Goat Anti-Rabbit IgG (Life Technologies) diluted 1:1000. Finally, cells were added with a drop of Fluoroshield™ (Sigma-Aldrich), mounted with a cover glass and scrutinized under a fluorescence light microscope (Olympus IX81).

Synthesis and characterization of reverse genetics viruses

Wild-type and chimeric RG betanodaviruses were generated by applying the principle of the infectious RNA transcription system developed by Iwamoto et al. [42]. Approximately 5 ng of pI18 plasmids containing the inserts of interest (Fig 1B) were digested at 37°C for 2 hours with *Eco*RI (New England Biolabs). Linear plasmids were subjected to electrophoresis in 1% agarose gel and were subsequently purified with the QIAquick Gel Extraction Kit (Qiagen) following manufacturer's recommendations. About 1 μg of linear plasmid template was transcribed into RNA using the mMessage mMachine® T7 Transcription Kit (Life Technologies), which incorporates a cap analogue at the 5' end of each genetic segment. The reaction mix was added with 40U of RNAsin® Plus RNase Inhibitor (Promega) and incubated at 37°C for 3 hours. The reaction was completed by 15 min incubation at 37°C with 1 μl TURBO DNase. Synthetic RNA was purified using the MEGAclear™ Transcription Clean-Up kit (Life Technologies) following the manufacturer's recommendations. Twenty-four hours before transfection, E-11 cells

Fig 1. Genetic structure of the RNA1 and the RNA2 inserts cloned for the expression of recombinant coat proteins in EPC cells (A) and for the generation of reverse genetics viruses (B). Thick bars represent RNA1 and RNA2 open reading frames (ORF), and thin bars indicate the 5'-UTR and 3'-UTR regions. Light grey designates nucleotide sequence of the RGNNV genotype (strain 283.2009), while dark grey indicates genetic sequence of the SJNNV genotype (strain 484.2.2009). Numbers specify the nucleotide position of the chimeric junction between the RGNNV and the SJNNV sequences. Numbers refer to the full length RNA2 nucleotide sequence related to strain 484.2.2009 (GenBank accession number JN189919).



(approximately 2.5 × 10^4/ml) were seeded in 24-well plates with complete medium. A total of 500 ng of RNA1 and 250 ng of RNA2 transcripts were transfected into E-11 cells with the Lipofectamine® MessengerMAX™ Transfection Reagent (ThermoFisher Scientific) according to the producer’s instructions. Cells were incubated at 25°C and checked daily for CPE appearance. Upon disruption of cell monolayers, culture fluids were collected, frozen and thawed, and then passaged into fresh E-11 cells. After complete CPE had occurred and viral progeny had multiplied, cell culture supernatants were subjected to transmission electron microscopy (TEM). Samples were prepared according to standard procedures, negative stained with 2% sodium phosphotungstate solution and finally observed with transmission electron microscope (Philips 208S) [43]. Reverse genetics viruses were subjected to sequencing and serologically characterized through SN in four independent replicates as described above, using sera anti-RGNNV and anti-SJNNV. Their neutralization titres were compared with those of wild-type RGNNV (283.2009) and SJNNV (484.2.2009) strains.

Results

Serological classification of fish nodaviruses

The rabbit antisera produced in this study showed high neutralizing titres against the homologous antigens, ranging from 1:1280 to 1:40960. Their specificities were tested against other fish viruses such as viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV). No cross-reactions were observed (data not shown).

The serological relationships among betanodaviruses with different genomes are described by the 1/r value, defined as the reciprocal of the geometric mean titre(GMT) of the two ratios obtained with the heterologous viruses and the homologous sera [37]. Larger 1/r values indicate greater serological differences between strains (Table 1). By the use of a cut-off value of 10 [38], viruses analysed in the present study can be divided into three different serological groups called A, B and C, in compliance with the nomenclature previously established by Mori et al. [29]. In detail, strains 484.2.2005 (SJNNV) and 367.2.2005 (RGNNV/SJNNV) form serogroup A (1/r = 2.07), while viruses 283.2009 (RGNNV) and 389/I96 (SJNNV/RGNNV) cluster within serogroup C (1/r = 2.54). The serotyping of the reassortant strains RGNNV/SJNNV and SJNNV/RGNNV has led to the

observation that the genetic type of the coat protein gene actually drives the immunoreactivity of these viruses. Differently from the serological categorization proposed by Mori and collaborators [29], strain JFIwa98 (BFNNV) does not belong to serogroup C, but can be classified as part of serogroup B together with strains TPKag93 (TPNNV), SK-07 1324 (BFNNV) and Ah95NorA (BFNNV) (1/r values ranging between 0.65 and 3.95). However, it must be mentioned that most of the 1/r values estimated among members of serogroups B and C are slightly above the threshold (i.e. 10.56 – 19.70), thus suggesting a certain level of cross-reactivity among genotypes RGNNV, SJNNV/RGNNV, TPNNV and BFNNV.

Statistical analyses

Spearman coefficients are reported in Table 1. A very strong correlation is observed between viruses of serotype A (484.2.2005 and 367.2.2005) and between viruses of serotype C (283.2009 and 389/I96). Strain 283.2009 is moderately correlated also with viruses 484.2.2005 and 367.2.2005. Viruses belonging to serotype B correlate with each other, and correlate also with strains of serotypes A and C. In detail, virus JFIwa98 shows a weak correlation with strain TPKag93 and a moderate correlation with virus SK-07 1324. This latter is also moderately correlated with virus 389/I96. Strain Ah95NorA shows a moderate correlation with viruses belonging to serotypes A and C (484.2.2009, 367.2.2005

Table 1. 1/r values and Spearman coefficients among different betanodavirus isolates.

On the right: Serological relationships of genetically different betanodaviruses expressed as 1/r values. Values < 10 (in bold) indicate that two viruses belong to the same serogroup. On the left: Spearman correlation coefficients between viruses, p-value and number of observations between brackets. Statistically significant correlations (p-value < 0.05) are highlighted in bold.

		1/r							
		283.2009	389/I96	484.2.2009	367.2.2005	JFIwa98	TPKag93	SK-07 1324	Ah95NorA
Spearman correlation coefficient	283.2009		**2,54**	53,82	59,71	19,70	17,75	10,56	16,95
	389/I96	**0,87 <0,0001 (32)**		173,99	99,93	47,43	17,97	23,72	17,16
	484.2.2009	**0,50 0,0038 (31)**	0,33 0,0688 (31)		**2,07**	84,45	34,90	51,98	33,90
	367.2.2005	**0,50 0,0053 (29)**	0,33 0,0728 (29)	**0,98 <0,0001 (29)**		119,43	49,35	48,50	59,03
	JFIwa98	0,25 0,1597 (32)	0,25 0,1592 (32)	-0,20 0,2609 (31)	-0,21 0,2580 (29)		**0,65**	**2,14**	**3,89**
	TPKag93	0,19 0,2728 (32)	-0,14 0,4353 (32)	**-0,40 0,0237 (31)**	**-0,40 0,0314 (29)**	**0,35 0,0492 (32)**		**0,75**	**1,26**
	SK-07 1324	0,34 0,0517 (32)	**0,40 0,0203 (32)**	-0,02 0,8892 (31)	-0,02 0,9161 (29)	**0,55 0,0010 (32)**	0,24 0,1772 (32)		**3,95**
	Ah95NorA	**0,46 0,0073 (32)**	0,25 0,1577 (32)	**0,49 0,0043 (31)**	**0,56 0,0015 (29)**	0,31 0,0751 (32)	-0,20 0,2616 (32)	-0,01 0,9185 (32)	

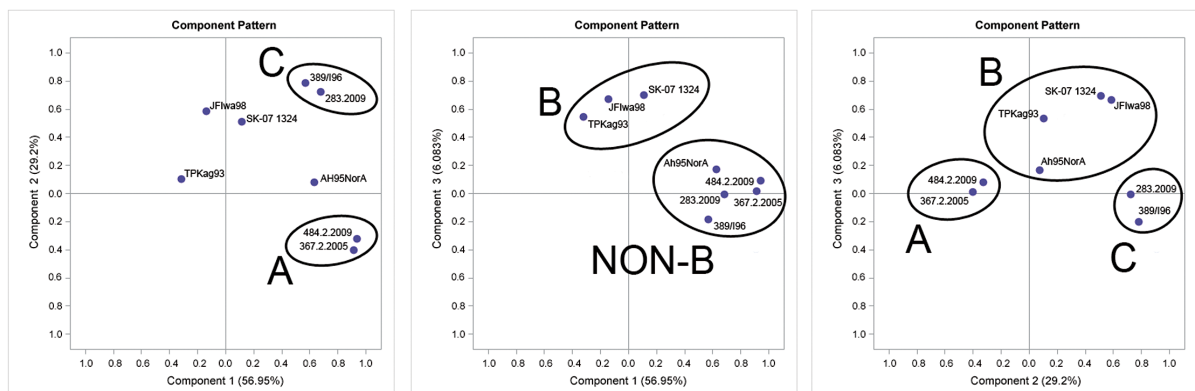
and 283.2009). Finally, strain TPKag93 is significantly uncorrelated with viruses of serotype A (484.2.2009 and 367.2.2005).

The scree plot and the eigenvalue obtained by the PCA analysis indicate that the first three principal components account for a meaningful amount of variance. The total explained variability by the first 3 PCs is 92% (56.95% for PC1; 29.2% for PC2; 6.08% for PC3) (S2 Figure). Viruses RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV identify the first component, while the second component arranges viruses into two different groups named A (SJNNV, RGNNV/SJNNV) and C (RGNNV and SJNNV/RGNNV). Viruses JFIwa98, TPKag93, SK-07 1324 and Ah95NorA identify the third principal component and cluster together within group B (Figure 2).

Serological typing of field isolates

To further evaluate the capability of the SN assay to recognize the serotype of unidentified samples, 11 field isolates were used as antigens and tested with the raised antisera. The neutralization titres obtained are shown in Table 2. The field strains clustered in the expected serotypes in accordance to their genotype. In detail, strains 132.2005, 82/I07 and

Fig 2. Clustering of viruses by pattern plots of the principal component (PC). The principal components analysis is based on the serum neutralization titres expressed as GMT of the isolates tested. In each graphic, the three PCs are plotted one against the other. Viruses are grouped into three different clusters named A, B and C.



250.1.2009 (reassortants RGNNV/SJNNV) were classified as serotype A, while isolates 512.2000, 390.3.2003, 80.1.5.2005, 498.2.2005, 550.2.2005, 320.1.2009, 396.3.2011 and E.marginatus/I/35-1/Dec13 (RGNNV) fell within serotype C.

Recombinant capsid proteins expression and immunostaining

RGNNV, SJNNV and chimeric capsid proteins A, B, C, D, E and F were successfully expressed in EPC cells, and antigenically characterized with anti-RGNNV and anti-SJNNV (Fig 3). A moderate level of cytotoxicity was observed in EPC cells overexpressing recombinant capsid proteins, while non-transfected cells appeared unaltered (data not shown). Staining using the anti-RGNNV serum was observed for cells transfected with RGNNV CP, and chimeric capsid proteins B, C and F. RGNNV and chimeric capsid protein C and F had both nuclear and cytosolic staining, while chimeric protein B showed only nuclear staining. A significantly lower transfection efficiency was observed for chimeric protein F. The RNA2 sequences encoding for capsid proteins RGNNV, B, C and F share the RGNNV nucleotide sequence 676-795 (aa 217-256). In EPC cells expressing chimeric capsid proteins D and E, anti-RGNNV stained weakly a few nuclei with a granular pattern. Serum anti-SJNNV recognized SJNNV CP and chimeric capsid protein A and C, a granular pattern in some nuclei could be seen in A. The RNA2 sequences encoding for these proteins contain the SJNNV nucleotide sequence 796-1023 (aa 257-341). No fluorescent signal was observed in EPC cells stained with serum anti-SJNNV expressing capsid proteins RGNNV, B, D, E and F. Notably, chimeric capsid protein C was recognized by both sera anti-RGNNV and anti-SJNNV.

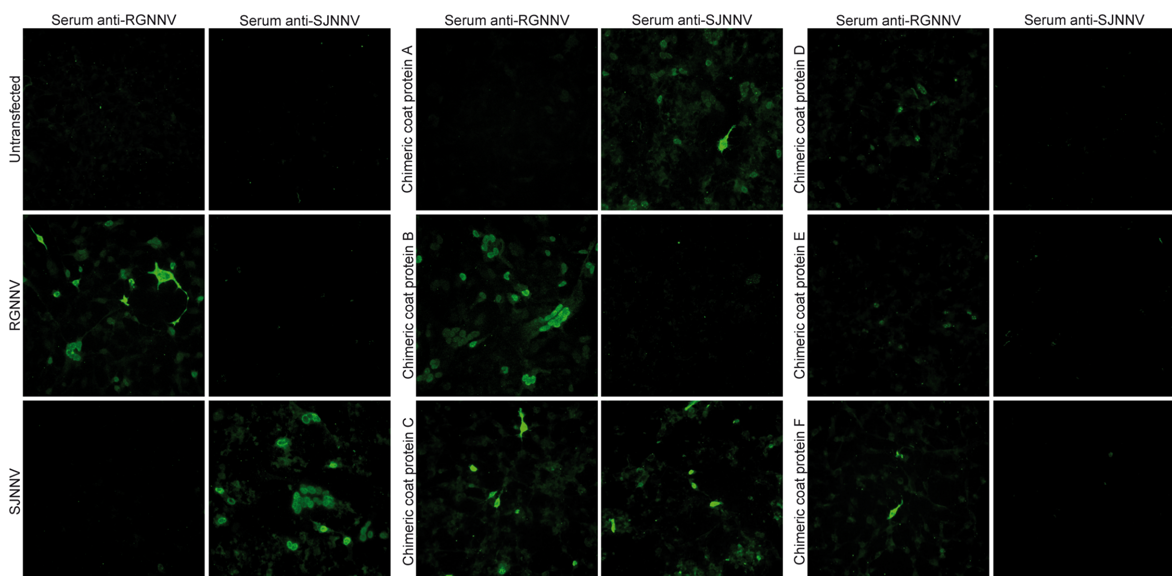
Table 2. Serological typing of field isolates. Results of serum neutralization tests are expressed as neutralizing titres using rabbit anti-sera against different betanodavirus genotypes.

	Rabbit antisera							
Field isolates (genotype)	Serum anti-283.2009	Serum anti anti-389/I96	Serum anti-484.2.2009	Serum anti-367.2.2005	Serum anti-JFIwa98	Serum anti-SK-07 1324	Serum anti-TPKag93	Serum anti-Ah95NorA
512.2000 (RGNNV)	1:10240	1:640	1:10	1:320	1:10	1:40	1:160	1:10
390.3.2003 (RGNNV)	1:20480	1:1280	1:10	1:640	1:160	1:160	1:160	1:10
80.1.5.2005 (RGNNV)	1:20480	1:1280	1:40	1:640	1:160	1:160	1:160	1:20
498.2.2005 (RGNNV)	1:10240	1:640	1:10	1:320	1:80	1:80	1:40	1:80
550.2.2005 (RGNNV)	1:10240	1:640	1:40	1:640	1:40	1:160	1:80	1:80
320.1.2009 (RGNNV)	1:10240	1:2560	1:80	1:640	1:80	1:160	1:40	1:10
396.3.2011 (RGNNV)	1:10240	1:2560	1:160	1:1280	1:80	1:80	1:2560	1:20
E.marginatus/I/35-1/Dec13 (RGNNV)	1:5120	1:1280	1:160	1:640	1:40	1:40	1:640	1:10
132.2005 (RGNNV/SJNNV)	1:160	1:80	1:10240	1:20480	1:10	1:10	1:20	1:80
82/I07 (RGNNV/SJNNV)	1:640	1:160	1:2560	1:5120	1:10	1:10	1:10	1:320
250.1.2009 (RGNNV/SJNNV)	1:160	1:80	1:10240	1:20480	1:10	1:10	1:20	1:640

Recovery and characterization of reverse genetics viruses

The generation of progeny viruses from infectious transcripts was determined by CPE formation and TEM observation. At 24 hours post transfection (h.p.t.), typical foci of rounded, granular and vacuolated cells were observed in all samples. At 48 h.p.t., cell monolayers were completely disrupted. E-11 cells inoculated with Lipofectamine® MessengerMAX™ Transfection Reagent only, showed no cytotoxicity. Only the RGNNV RG, the SJNNV RG, chimera-A RG and chimera-B RG replicated and were able to produce typical CPE after passage to new E-11 monolayers. When subjected to TEM, virions compatible in both size and shape with betanodavirus particles were observed for these samples only (Fig 4).

Fig 3. EPC cells expressing RGNNV, SJNNV and chimeric capsid proteins A, B, C, D, E and F. Cells were immunostained with serum anti-283.2009 (RGNNV) and anti-484.2.2009 (SJNNV). Images were taken at 20x magnification.



The recovered RG viruses and the wild-type RGNNV and SJNNV were subjected to SN with anti-RGNNV and anti-SJNNV (Table 3). Viruses RGNNV wild-type (283.2009) and RGNNV RG were specifically neutralized by anti-RGNNV (1:10240 and 1:1280, respectively), while their SN with anti-SJNNV was negligible (1:10). On the other hand, anti-SJNNV strongly neutralized SJNNV wild-type (484.2.2009) (1:20480) and SJNNV RG (1:5120/1:10240), however, these were also slightly neutralized by anti-RGNNV

(1:320/1:640 and 1:640, respectively). Remarkably, data obtained from the characterization of chimera-A RG and chimera-B RG indicate that their serological profiles are primarily determined by the C-terminus of their coat proteins. Indeed, chimera-A RG was strongly neutralized by anti-SJNNV, with neutralizing titre similar to that obtained for the SJNNV wild type (1:20480). Chimera-A RG was also neutralized by anti-RGNNV, but at significantly lower extent (1:2560/1:5120). Conversely, chimera-B RG was strongly neutralized by anti-RGNNV, with neutralizing titre identical to that of the RGNNV wild type (1:10240), while it was barely neutralized by anti-SJNNV (1:160/1:320).

Fig 4. Negative staining TEM of recombinant viruses obtained through the reverse genetics technology. Pictures were taken at 28x magnification.

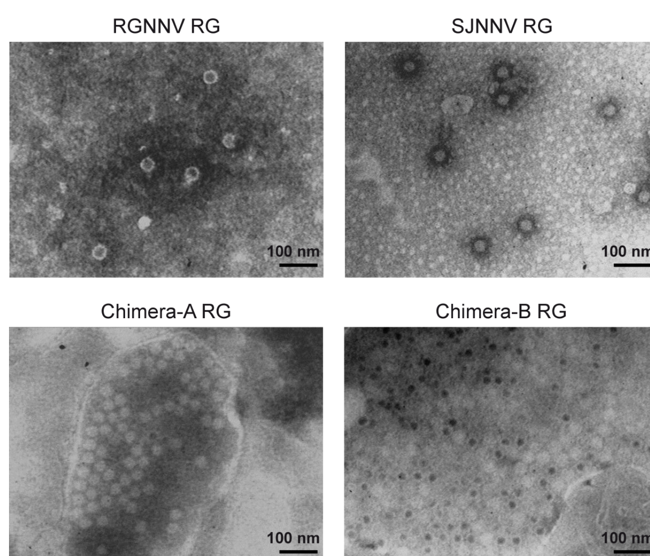


Table 3. Serum neutralization tests of wild type and recombinant viruses. Wild type strains 283.2009 (RGNNV) and 484.2.2009 (SJNNV), and recombinant viruses RGNNV RG, SJNNV RG, chimera-A RG and chimera-B RG were subjected to serum neutralization using rabbit hyperimmune sera anti-283.2009 and anti-484.2.2009. SNs were carried out in four replicates. Results are expressed as neutralizing titres.

	Serum anti-283.2009	**Serum anti-484.2.2009**
283.2009 wild-type	1:10240	1:10
484.2.2009 wild-type	1:320/1:640	1:20480
RGNNV RG	1:1280	1:10
SJNNV RG	1:640	1:5120/1:10240
Chimera-A RG	1:2560/1:5120	1:20480
Chimera-B RG	1:10240	1:160/1:320

Discussion

The study of the immunoreactivity and the immunogenicity of fish pathogens is required for developing serological diagnostic tests and for designing vaccines. Information on the antigenic properties is particularly scarce for the fish nodaviruses, and currently there is only one paper providing serological characterization of different betanodavirus genotypes, that originated mostly from Asia [29]. The serological classification proposed was used in our study but cold-water strains, the newly emerged reassortants and viruses from the Mediterranean basin were implemented. Our results showed that betanodaviruses can be classified into 3 different serogroups, namely A, B and C, in conformity with the nomenclature previously established. Serotype A comprises SJNNV and RGNNV/SJNNV viruses, while serotype C consists of RGNNV and SJNNV/RGNNV strains. Differently from what previously reported, our data show that the BFNNV genotype clusters together with the TPNNV virus and the cold water isolates within serotype B. In their work, Mori and colleagues had used a threshold of 20 for the 1/r index to establish whether two different viruses belonged to same serogroup. According to this criterion, they categorized the BFNNV genotype within serotype C, although they had also observed an exceptional cross-reactivity with the TPNNV genotype (1/r = 16) [29]. In the present study, we have set up a lower threshold for the 1/r index (i.e. 10), as adopted by Hill and Way [38] for the serological categorization of aquatic *Birnaviruses*. This choice was further supported by the PCA that clearly identified 3 clusters of viruses: A (SJNNV, RGNNV/SJNNV), B (BFNNV, TPNNV) and C (RGNNV, SJNNV/RGNNV) (Fig 2). However, it must be mentioned that some of the 1/r values calculated among isolates of serogroup B are slightly above the threshold (10 < 1/r < 20), and the Spearman coefficients calculated for these viruses highlight for some of them a weak/moderate correlation with strains belonging to serotypes A and C. Taken together, these data suggests that viruses belonging to serotype B are partially cross-reactive with viruses belonging the other serotypes.

The antigenic differences between the RGNNV and the SJNNV genotypes have been assessed by several authors [29,30,44-46]. Consistent with their assumptions, we found that there is little or no cross-reactivity between betanodaviruses of serogroups A and C, as confirmed by the high 1/r values and statistical analyses. Furthermore, our data indicate that the serotype of the RGNNV/SJNNV and SJNNV/RGNNV reassortants is determined by the genotype of the donor for the RNA2 segment, i.e. the coat protein gene is the major

genetic determinant of betanodavirus immunoreactivity. Tang et al. [21] reconstructed the 3D structure of the malabaricus grouper nervous necrosis virus VLPs, suggesting that residues 217-388 form a protruding domain located at the outer surface of the capsid. Similarly, Chen et al. [22] described the crystal structure of GNNV-LP and identified a protrusion domain (aa 214-388) putatively involved in the calcium-mediated trimerization of the capsid proteins during the inner phases of betanodavirus capsid assembly. Besides, the authors observed that a number of hypervariable regions of the P-domain overlap with functional regions implicated in cell receptor binding and species tropism [23]. Accordingly, we hypothesized that the protrusion domain might also be involved in other host-pathogen interaction processes, such as antibody recognition. Interestingly, data obtained from the immunostaining of chimeric coat proteins A and B demonstrate that residues from aa 217 to the C-terminus retain the immunoreactive portion of the RGNNV and the SJNNV capsid proteins, in concordance with the 3D structural models proposed by Tang et al. and Chen et al. [21,22]. It is also noteworthy that SN tests carried out for chimera-A RG and chimera-B RG indicate that the epitopes comprised between aa 217 and the C-terminus of their coat proteins contain the neutralizing epitopes. Remarkably, the immunostaining patterns of EPC cells expressing the various chimeric capsid proteins indicate that the immunoreactive residues of the RGNNV and the SJNNV genotypes are located at different positions of the coat protein sequence. Proteins retaining the RGNNV aa residues 217-256 were recognized by anti-RGNNV, while proteins containing the SJNNV aa sequence 257-341 reacted with anti-SJNNV. Immunoblotting partial coat proteins of the four known species with SJNNV specific MAbs, Nishizawa et al. [30] pinpointed the $PAN_{254\text{-}256}$ motif of the SJNNV capsid protein as a candidate neutralizing epitope for this genotype. In the present study, the absence of staining with serum anti-SJNNV of chimeric proteins D and E, which contain the $PAN_{254\text{-}256}$ motif, contradicts this finding. Such a result might be due to the fact that the $PAN_{254\text{-}256}$ motif is a linear epitope, and therefore it might not be accessible to antibody recognition using serum anti-484.2.2009. On the other hand, Iwamoto and colleagues [47] reported that putative bulging positions in the 236-338 region of the SJNNV and the RGNNV CPs show noticeable differences in their surface probability scores, suggesting the presence of structural variations between the capsid proteins of these two genotypes. This observation might corroborate the hypothesis that the immunoreactive epitopes of the RGNNV and the

SJNNV genotypes are situated at different positions in the coat protein sequence. Interestingly, Ito and colleagues [23] identified the nucleotide regions 694-758 for the RGNNV and 695-765 for the SJNNV as the molecular determinants controlling species tropism. Notably, the RNA2 sequence regulating the RGNNV host specificity is comprised within the nucleotide region 675-795 determining also the immunoreactivity for this genotype. On the contrary, the capsid protein portions involved in host tropism and antibody recognition of the SJNNV are encoded by different nucleotide regions (nt 695-765 and nt796-1023, respectively). It cannot be ruled out that the structural and functional differences observed between the RGNNV and the SJNNV might be responsible for the diverse phenotypes in terms of species specificity and pathogenicity observed for these genotypes.

Data resulting from the immunostaining of chimeric coat proteins were substantiated only in part through the serological neutralization test of the reverse genetics derived viruses. We were unable to obtain chimera-C RG, chimera-D RG, chimera-E RG and chimera-F RG, despite we observed complete CPE at 48 hpt for these viruses. It is noteworthy that the chimeric constructs C, D, E and F present a junction between the RGNNV and the SJNNV sequences at the nucleotide position 795. We hypothesize that such a genetic manipulation might have generated defective particles unable to replicate due to the interruption of genetic signatures that are critical for viral viability, for instance recruiting signal for the formation of the replication complex, packaging signal, capsid assembly signal, protein localization signal, etc. Further investigations are required to explore the role of this for betanodavirus biology and fitness.

In summary, in the present study we have characterised some determinants responsible for betanodavirus immunoreactivity including an overview of the serological relationship among different genotypes. The results show that serological characterization is a good alternative to genetic typing of betanodaviruses. Importantly, field isolates were grouped consistently by SN test and genotyping, thus validating the SN test as an specific method for routine diagnosis and typing. Indeed, the low cross-reactions among different betanodavirus serotypes indicate that the SN testcould be useful for virus characterization in areas where more than one betanodavirus genotype is circulating, such as the Mediterranean basin [27]. It also indicates that monovalent vaccines will not be sufficient

to obtain protection, which may be important information for future development of vaccines against VNN caused by any betanodavirus genotype.

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Supplementary material

S1 Table. List of betanodavirus strains used in the present study.

Strain	Genotype	Host	Origin	RNA2 Acc. No.	Reference
283.2009*	RGNNV	*Dicentrarchus labrax*	Italy	JN189992	[27]
484.2.2009*	SJNNV	*Solea senegalensis*	Spain	JN189919	[27]
367.2.2005*	RGNNV/SJNNV	*Dicentrarchus labrax*	Italy	JN189936	[27]
389/I96*	SJNNV/RGNNV	*Dicentrarchus labrax*	Italy	KF386164	[48]
JFIwa98*	BFNNV	*Paralichthys olivaceus*	Japan	EU236147	[49]
TPKag93*	TPNNV	*Takifugu rubripes*	Japan	EU236149	[49]
SK-07 1324*	BFNNV	*Gadus morhua*	Norway	KU355847	[50]
Ah95NorA*	BFNNV	*Hippoglossus hippoglossus*	Norway	KU355849	[51]
512.2000**	RGNNV	*Dicentrarchus labrax*	Italy	JN190031	[27]
390.3.2003**	RGNNV	*Solea solea*	Italy	JN190024	[27]
80.1.5.2005**	RGNNV	*Dicentrarchus labrax*	Italy	JN190033	[27]
498.2.2005**	RGNNV	*Mullus barbatus*	Italy	JN190016	[27]
550.2.2005**	RGNNV	*Epinephelus spp.*	Greece	JN189975	[27]
320.1.2009**	RGNNV	*Dicentrarchus labrax*	Spain	JN189982	[27]
396.3.2011**	RGNNV	*Epinephelus costae*	Italy	JX290533	[51]
E.marginatus/I/35-1/Dec13**	RGNNV	*Epinephelus marginatus*	Italy	KU355848	This study
132.2005**	RGNNV/SJNNV	*Dicentrarchus labrax*	Italy	JN189937	[27]
82/I07**	RGNNV/SJNNV	*Sparus aurata*	Italy	JX290518	[48]
250.1.2009**	RGNNV/SJNNV	*Sparus aurata*	Cyprus	JN189920	[27]

*Viral isolates used for rabbit hyperimmune sera production and serological classification of fish nodaviruses

**Unknown field isolates used for blind evaluation of the SN test

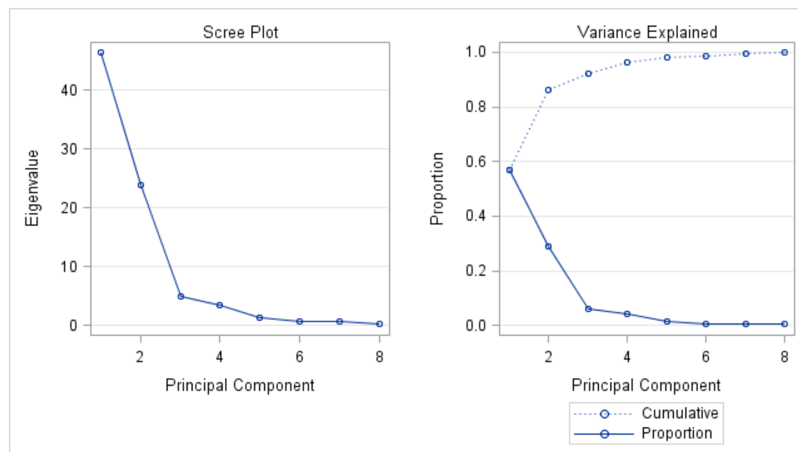
S2 Table. Pairwise nucleotide distances estimated among the RNA2 sequences of the betanodaviral strains used in the present study.

	283.2009	484.2.2009	367.2.2005	389/I96	JFIwa98	TPKag93	SK-07_1324	Ah95NorA	512.2000	390.3.2003	80.1.5.2005	498.2.2005	550.2.2005	320.1.2009	396.3.2	E.marginatus/I/35-1/Dec13	132.2005	82/I07	250.1.2009
283.2009	0.0																		
484.2.2009	0.30																		
367.2.2005	0.30	0.04																	
389/I96	0.20	0.35	0.34																
JFIwa98	0.25	0.29	0.32	0.27															
TPKag93	0.29	0.24	0.23	0.33	0.26														
SK-07_1324	0.25	0.29	0.32	0.27	0.02	0.26													
Ah95NorA	0.26	0.29	0.32	0.28	0.02	0.28	0.03												
512.2000	0.03	0.31	0.30	0.20	0.27	0.29	0.25	0.27											
390.3.2003	0.07	0.30	0.28	0.19	0.23	0.29	0.23	0.24	0.06										
80.1.5.2005	0.06	0.30	0.29	0.18	0.24	0.29	0.23	0.24	0.05	0.03									
498.2.2005	0.08	0.30	0.29	0.21	0.22	0.29	0.22	0.22	0.07	0.04	0.05								
550.2.2005	0.06	0.32	0.30	0.20	0.23	0.30	0.23	0.23	0.07	0.03	0.05	0.03							
320.1.2009	0.08	0.30	0.29	0.21	0.21	0.30	0.23	0.22	0.08	0.04	0.05	0.02	0.03						
396.3.2011	0.07	0.32	0.29	0.19	0.22	0.30	0.23	0.23	0.06	0.03	0.03	0.02	0.02	0.02					
E.marginatus/I/35-1/Dec13	0.07	0.32	0.31	0.21	0.23	0.31	0.23	0.23	0.07	0.03	0.05	0.03	0.01	0.02	0.02				
132.2005	0.31	0.04	0.01	0.36	0.32	0.24	0.33	0.32	0.31	0.29	0.30	0.30	0.31	0.29	0.30	0.31			
82/I07	0.30	0.04	0.00	0.34	0.31	0.23	0.32	0.32	0.30	0.28	0.29	0.29	0.30	0.29	0.29	0.31	0.01		
250.1.2009	0.30	0.04	0.01	0.34	0.31	0.22	0.31	0.31	0.30	0.28	0.29	0.29	0.30	0.28	0.29	0.30	0.02	0.01	0.0

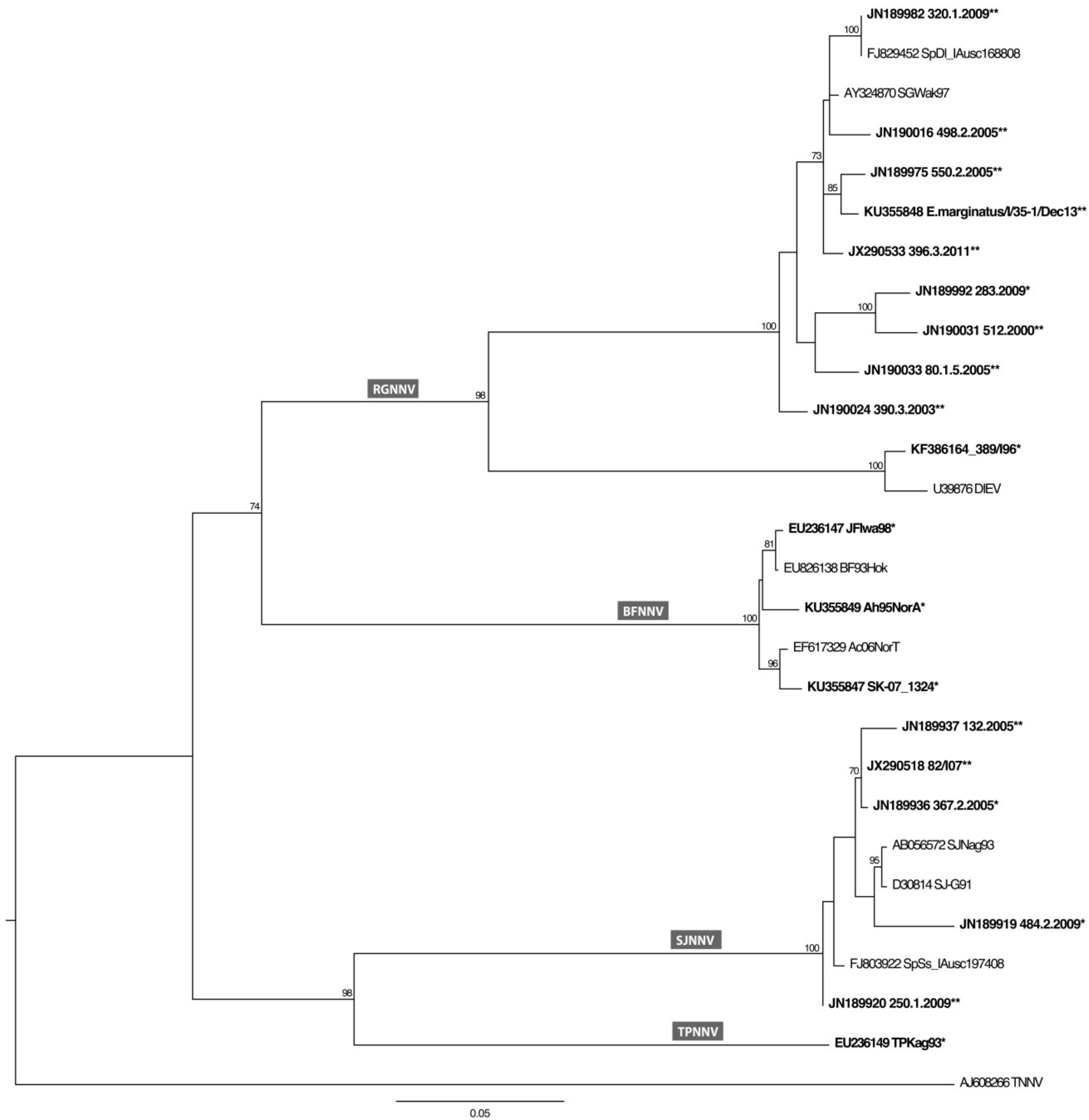
S3 Table. Primers adopted for the In-Fusion reactions. The RGNNV and the SJNNV specific sequences are underlined with dashed line and double line, respectively. The T7 promoter sequence is indicated in lowercase. Restriction enzyme sites are showed in italics (BamHI: GGATCC; EcoRI: GAATTC; KpnI: GGTACC; BglII: AGATCT). pcDNATM3.1(+) and pI18 specific sequences are indicated with continuous line and dot dash line, respectively.

Primer	Sequence 5'? 3'
RNA1	
pI18_RdRp_RG_5'UTR_F	TCCTTGACACGATC*GGATCC*taatacgactcactatagTAACATCAGCTCTTGCTCTG
pI18_RdRp_RG_3'UTR_R	TGACGTGGGATCTA*GAATTC*GCCGAAGCGTAAGACAGCA
pI18_RdRp_SJ_5'UTR_F	TCCTTGACACGATC*GGATCC*taatacgactcactatagTAACATCACCTTCTTGCT
pI18_RdRp_SJ_3'UTR_R	TGACGTGGGATCTA*GAATTC*GCCGAAGCGTAGGACAGCA
RNA2	
pcDNA3.1_RG_N-ter_F	TTAAACTTAAGCTT*GGTACC*ATGGTACGCAAAGGTGAGAAGAAATTGG
pcDNA3.1_RG_C-ter_R	ATCCGAGCTC*GGTACC*TTAGTTTTCCGAGTCAACCCTGGTGC
pcDNA3.1_SJ_N-ter_F	TTAAACTTAAGCTT*GGTACC*ATGGTACGCAAAGGTGATAAGAAATTGGCA
pcDNA3.1_484_C-ter_R	ATCCGAGCTC*GGTACC*TTAGTTTTCCGAGTCAACACGGGTGAAG
pI18_283_5'UTR_F	TACCTCTAGA*AGATCt*aatacgactcactatagTAATCCATCACCGCTTTGCAATCACAATG
pI18_283_3'UTR_R	GTGGGATCTA*GAATTC*GCCGAGTTGAGAAGCGATCAGC
pI18_484_5'UTR_F	TACCTCTAGA*AGATCt*aatacgactcactatagTAATCTAACACCGCTTTGCAAGTCAAAATGG
pI18_484_3'UTR_R	GTGGGATCTA*GAATTC*GCCGAGTATTGTAGCGATCAGCG
675_RG/SJjunct_R	TGGAGCGGTGGTATCTTCAGGTGTTTCAAGAGACGGAACGC
675_RG/SJjunct_F	CTTGAAACACCTGAAGATACCACCGCTCCAATCGCTAC
675_SJ/RGjunct_R	GGGAGCGGTGGTCTCCTCAGGTGTCTCGAGGGACG
675_SJ/RGjunct_F	CTCGAGACACCTGAGGAGACCACCGCTCCCATCATGA
795_RG/SJjunct_R	AGTGACATAGACAGCTCCGTCAGGAGCAATGTCCAGT
795_RG/SJjunct_F	ATTGCTCCTGACGGAGCTGTCTATGTCACTGACAAGCCGTT
795_SJ/RGjunct_R	CAGCTGGAAGATTGCGTTTGCAGGGGCGAGGTCG
795_SJ/RGjunct_F	CTCGCCCCTGCAAACGCAATCTTCCAGCTGGACCGT

S1 Figure. PCA scree plot and explained variance. The scree plot on the left shows the eigenvalues versus the number of the components. The point where the slope of the curve is levels off (the "elbow") indicates the number of components that have to be considered. The first three eigenvalues are identified and they are largely over 1. On the right, the plot shows that nearly the 92% of the total variance can be explained with the first three principal components.



S2 Figure. Phylogenetic tree of the betanodavirus isolates used in the present study. Partial RNA2 sequences related to the betanodaviral strains under investigation were aligned and compared with reference sequences available in GenBank. The phylogenetic tree was inferred using the maximum likelihood method (ML) available in the RaxML program, incorporating the GTR model of nucleotide substitution with the CAT model of rate heterogeneity among sites. To assess the robustness of individual nodes, 100 bootstrap replicates were performed. Betanodavirus isolates used in the present study are highlighted in bold. *Viral isolates used for rabbit hyperimmune sera production and serological classification of fish nodaviruses. **Unknown field isolates used for blind evaluation of the SN test.



Chapter 7

In vitro study of the replication capacity of the RGNNV and the SJNNV betanodavirus genotypes and their natural reassortants in response to temperature

Valentina Panzarin, Elisabetta Cappellozza, Marzia Mancin, Adelaide Milani, Anna Toffan, Terregino Calogero, Giovanni Cattoli

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In vitro study of the replication capacity of the RGNNV and the SJNNV betanodavirus genotypes and their natural reassortants in response to temperature

Valentina Panzarin*, Elisabetta Cappellozza, Marzia Mancin, Adelaide Milani, Anna Toffan, Calogero Terregino and Giovanni Cattoli

Abstract

Betanodaviruses are the causative agents of viral nervous necrosis and affect a broad range of fish species worldwide. Their bi-segmented genome is composed of the RNA1 and the RNA2 molecules encoding the viral polymerase and the coat protein, respectively. In southern Europe the presence of the RGNNV and the SJNNV genotypes, and the RGNNV/SJNNV and RGNNV/SJNNV reassortants has been documented. Several studies have reported a correlation between water temperature and disease onset. To explore the replication efficiency of betanodaviruses with different genomes in relation to temperature and to understand the role of genetic reassortment on viral phenotype, RGNNV, SJNNV, RGNNV/SJNNV and RGNNV/SJNNV field isolates were fully sequenced, and growth curves generated in vitro at four different temperatures (15, 20, 25, 30 °C) were developed for each isolate. The data obtained, corroborated by statistical analysis, demonstrated that viral titres of diverse betanodavirus genotypes varied significantly in relation to the incubation temperature of the culture. In particular, at 30 °C betanodaviruses under investigation presented different phenotypes, and viruses containing the RNA1 of the RGNNV genotype showed the best replication efficiency. Laboratory results demonstrated that viruses clustering within the same genotype based on the polymerase gene, possess similar growth kinetics in response to temperature, thus highlighting the key role of RNA1 in controlling viral replication at different environmental conditions. The results generated might have practical implications for the inference of viral phenotype according to genetic features and may contribute to a better understanding of betanodavirus ecology.

Introduction

The increasing amount of genetic information obtained from viral genomes sequencing, aids research into the exploration of the genotype-phenotype relationships to determine the genetic traits responsible for different phenotypic features, which in turn may have practical implications for disease recognition and control. Nevertheless, the phenotype of a virus is not only dependent on its "intrinsic" genetic features (e.g. specific mutations, reassortment, recombination) but it is also governed by "extrinsic" variables [1-4]. Among these, temperature is certainly one of the most important environmental factors in determining the ecological and physiological status of viruses hosted by poikilothermic animals such as fish, whose thermoregulation systems are generally absent or extremely rudimentary. Fish body temperature varies with that of their environment, and consequently aquatic viruses need to adapt to a wide range of temperatures to be able to replicate at different conditions.

Viruses within the genus *Betanodavirus* are the causative agents of a highly infectious fish disease known as viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER). The genome of betanodaviruses is composed of two single stranded positive sense RNA molecules. The RNA1 segment encodes the RNA-dependant RNA polymerase (RdRp), or protein A, and

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gives rise to the RNA3 sub-genomic transcript which is translated into proteins B1 and B2, whilst the RNA2 segment encodes the coat protein (CP) [5-8]. The phylogenetic analysis of the RNA2 genetic segment allowed the identification of four different genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), red-spotted grouper nervous necrosis virus (RGNNV) [9]. A putative fifth genotype isolated from *Scophthalmus maximus* named as turbot nervous necrosis virus (TNNV) is awaiting for official classification [10]. Iwamoto et al. [11] demonstrated that the degree of viral replication in SSN-1 monolayers and the severity of the cytopathic effect (CPE) may vary according to the genotype and to the incubation temperature of the cultures. It has been also observed that optimal culture temperatures vary among genotypes: 15–20 °C for the BFNNV genotype, 20 °C for the TPNNV genotype, 20–25 °C for the SJNNV genotype and 25–30 °C for the RGNNV genotype [12]. More recent studies have highlighted that it is the RGNNV genotype that can replicate in vitro at the widest range of temperatures, from a minimum of 15 °C to a maximum of 35 °C [13,14]. Noteworthy, betanodaviruses are widely distributed worldwide in cold, temperate and tropical climate zones. Generally speaking, temperature dependency of betanodaviruses seems to correspond to their geographic distribution. To date, the TPNNV genotype has been described only once in Japan [9]. Cold water betanodaviruses grouping within the BFNNV genotype have been reported in Norway, France, the UK, eastern Canada and in the north-east of the USA [15-21]. The SJNNV genotype distribution appears confined to Spain and Japan [9,22-25]. Conversely, as a result of viral adaptation to different temperatures, the RGNNV seems to be the most widely diffused genotype, extending to Asia, Africa, Australia and several other Mediterranean areas and, accordingly, it is able to infect a large variety of warm water finfish species [23,24,26-33]. Together with the RGNNV and the SJNNV genotypes, the circulation of reassortant viruses in the form of the RGNNV/SJNNV, harbouring the RNA1 of the RGNNV and the RNA2 of the SJNNV, and the SJNNV/RGNNV, harbouring the SJNNV-RNA1 and the RGNNV-RNA2, have also been reported in southern Europe [23,29,32]. To date, the biological and ecological properties of these viruses have been poorly described, and little is known about the role of genetic reassortment and its effects on viral phenotype.

In order to identify the genetic regions involved in temperature dependency of piscine nodaviruses, the infectious RNA transcription system established by Iwamoto et al. [34] has recently been applied to produce artificial RGNNV and SJNNV viruses and their reassortants. The study has demonstrated that both genetic segments are involved in determining temperature sensitivity of betanodaviruses; however, the RNA1 molecule is capable of regulating this process independently from RNA2, confirming the observation that viral replication is vulnerable to temperature variations [35]. Although the reverse genetics technology provides a suitable experimental model for studying reassortant betanodaviruses, no information is available for natural field strains. For the first time, we have investigated the role of genetic reassortment in naïve betanodaviruses, as well as the genotype-phenotype relation as a function of temperature. For this purpose, natural reassortant RGNNV/SJNNV and SJNNV/RGNNV strains and RGNNV and SJNNV genotypes were genetically characterized and cultivated in cell monolayers at different incubation temperatures to assess their replication efficiency. Experimental data were validated through extensive statistical analysis.

Materials and methods

Virus strains and propagation in cell culture

On the basis of previous phylogenetic analysis of partial RNA1 and RNA2 sequences [23,36], four betanodavirus isolates representative of the RGNNV (283.2009), SJNNV (484.2.2009), RGNNV/SJNNV (367.2.2005) and SJNNV/RGNNV (389/I96) genetic variants were selected for further genetic and phenotypic characterization (Table 1). All but one of the selected viruses (283.2009; 367.2.2005; 389/I96) were originally isolated from the same fish species (sea bass, *Dicentrarchus labrax*). However, strain 484.2.2009 isolated from a Senegalese sole (*Solea senegalensis*) was also included in the selection, because to date no SJNNV infection has been reported in sea bass.

Betanodavirus isolates were propagated in E-11 cell monolayers (ECACC no. 01110916; [12]) incubated at 25 °C in Leibovitz medium (L-15) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FCS, L-Glutamine (2 mM) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B) [37]. Inoculated cell cultures were checked daily for cytopathic effect (CPE). Supernatants were collected upon disruption of cell monolayers, clarified by centrifugation at 4000 × *g* for 15 min at 4 °C, and subsequently subjected to further investigations.

RNA isolation and identification of the 3′ and 5′ terminal sequences

For each virus, total RNA was extracted from 100 μL of cell culture supernatant using the NucleoSpin® RNA II (Macherey–Nagel GmbH & Co, Düren, Germany), and subsequently treated with Tobacco Acid Pyrophosphatase (TAP) (Epicentre Biotechnologies, Madison, WI, USA) for 5′-cap removal. The 3′ and 5′ ends were ligated with the T4 RNA Ligase 1 (New England BioLabs, Ipswich, MA, USA) in a 25 μL reaction mix. Briefly, 15 μL

Table 1 Betanodavirus isolates used in this study for genetic and phenotypic characterization

Isolate	Genotype	Host	Origin	Reference	GenBank accession no.	
					RNA1	RNA2
283.2009	RGNNV	*Dicentrarchus labrax*	Italy	[23]	JN189865	JN189992
484.2.2009	SJNNV	*Solea senegalensis*	Spain	[23]	JN189814	JN189919
367.2.2005	RGNNV/SJNNV	*Dicentrarchus labrax*	Italy	[23]	JN189909	JN189936
389/I96	SJNNV/RGNNV	*Dicentrarchus labrax*	Italy	[46]	KF386163	KF386164

of de-capped RNA were incubated for 5 min at 65 °C with 20U rRNasin RNase Inhibitor (Promega, Fitchburg, WI, USA), 1X T4 RNA Ligase Reaction Buffer, 10% dimethyl sulfoxide (DMSO) and water, and subsequently cooled on ice for 2 min. The reaction mix was then incubated for 60 min at 37 °C with 20U of rRNasin RNase Inhibitor (Promega) and 20U of T4 RNA Ligase 1 (New England BioLabs). Enzymes were inactivated by incubating the reaction mix for 10 min at 65 °C. Circular RNA was subjected to reverse transcription (RT) for cDNA synthesis by using the SuperScript® III Reverse Transcriptase (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions and adding 10% DMSO to the reaction mix. PCR was subsequently carried out in a final volume of 25 μL containing 2 μL of template cDNA, 1X Cloned Pfu DNA polymerase reaction buffer, 240 μM dNTPs, 1.25U Pfu Turbo DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) and 0.2 μM specific forward and reverse primer hybridizing respectively upstream the 3' end and downstream the 5' end. Primer sequences are available upon request. The cDNA was denaturated at 95 °C for 2 min, and 40 cycles of the following conditions were applied: denaturation at 95 °C for 45 s, annealing at 50 °C for 1 min, elongation at 72 °C for 2 min. The amplification was completed with 2 min elongation at 72 °C. PCR products were subjected to electrophoresis in agarose gel, and were subsequently purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The sequences of the 3'-5' junctions were generated using the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies). The products of sequencing reactions were cleaned up using the Performa DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD, USA) and sequenced in a 16-capillary ABI PRISM 3130×l Genetic Analyzer (Life Technologies). Sequencing data were assembled and edited with the SeqScape® software v2.5 (Life Technologies).

Complete genome sequencing and phylogenetic characterization

In order to obtain complete genetic data related to the four selected betanodavirus strains, the nucleotide sequences corresponding to the full length RNA1 and RNA2 were determined for each virus. Previously de-capped RNA was subjected to RT by using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturer's instructions. The identification of the 3' and 5' terminal sequences allowed to design of specific primer sets for the whole genome amplification. PCR and sequencing were carried out as described above. Primer sequences can be provided on request.

The obtained sequences were aligned and compared to reference betanodavirus sequences publicly available in GenBank using the MEGA 4 package [38]. For both the RNA1 and RNA2 genetic segments, maximum likelihood (ML) phylogenetic trees were estimated using the best-fit general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites, and a heuristic SPR branch swapping search available in PhyML version 3.0 [39]. Bootstrap resampling (100 replications) assessed the robustness of individual nodes of the phylogeny. Pairwise nucleotide identities estimated among the RNA1 and RNA2 of the four betanodavirus strains herein described and the two reference strains SGWak97 [GenBank: AY324869; AY324870] and SJNag93 [GenBank: AB056571; AB056572] were determined.

Experimental design

E-11 cells were grown in 5 mL culture flasks with Leibovitz medium (L-15) (Sigma-Aldrich), supplemented with 10% FCS, L-Glutamine (2 mM) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin and 0,25 μg/mL Amphotericin B). Cell monolayers were infected in three replicates with each of the isolates at a multiplicity of infection (MOI) of 1.0. After 1 h adsorption at 25 °C, all the *inocula* were brought to the same volume with L-15 medium without growth factor (FCS). Inoculated monolayers were then incubated at 15, 20, 25 and 30 °C and checked regularly for CPE. A volume of 700 μL was sampled from each flask at 0 (T_0), 20 (T_1), 30 (T_2), 45 (T_3), 55 (T_4), 69 (T_5), 79 (T_6), 93 (T_7), 117 (T_8), 141 (T_9) and 165 (T_{10}) hours post infection (hpi), and subsequently replaced with an equal amount of medium. Collected cell culture supernatants were subjected to viral titration in E-11 monolayers by endpoint dilutions assays. Viral titres were calculated according to the Spearman-Karber formula [40]. Finally, average titres expressed as $TCID_{50}$/mL were calculated among replicates, and were used for developing growth curves for each incubation temperature.

Statistical analysis

The linear mixed model (LMM) for longitudinal data [41] was used to investigate the influence of genotype, temperature, exposure time and their possible interactions on viral replication efficiency. Different types of models with fixed and random effects were analysed according to the experimental design. To assess the repeatability of the experiment, replicates were tested as fixed effect, in addition to genotype, exposure time, temperature and their interactions. In this model it was assumed that each replicate was independent. Secondly, a linear mixed model was tested, where genotype, exposure time, temperature and their interaction were considered as fixed effects, while replicate was considered as a random effect. This model assumed constant variance among replicates of the same virus and among observations within the same replicate. The latter model was further developed considering different structures of variance and covariance matrix (VAR/COV) to take into account the existence of possible correlations (ρ) among repeated measurements of the same replicate. The structures of VAR/COV were: compound symmetry (CS), heterogeneous compound symmetry (CSH), unstructured (UN), first order autoregressive (AR (1)). *P*-values < 0.10 were considered significant. Graphical Student residuals analysis (residuals versus predicted plot, residuals distribution and residuals Q-Q plot) and tests for residuals normality (Shapiro Wilks and Kolmogorov Smirnov tests) were used to verify the goodness of the proposed models [41]. SAS software 9.3 was used to carry out the statistical analyses (PROC MIXED function) [42,43].

Results

Genetic characterization and phylogenetic analysis

The RNA1 genetic segment differed in length among the four betanodavirus strains. The 5'-untranslated regions (UTRs) were 78 nt long, whereas the 3'-UTRs varied from 75 to 77 nt. Strains 283.2009 and 367.2.2005 showed a 2949 nt ORF, corresponding to 983 aa, while the RNA1 coding region of samples 484.2.2009 and 389/I96 was 3 nt longer, determining an amino acid insertion in the RdRp at position 888. The RNA2 5'-UTRs and 3'-UTRs varied from 26 to 27 nt and from 370 to 390 nt, respectively. The complete ORF for strains 283.2009 and 389/I96 was 1017 nt, encoding a coat protein of 339 amino acids. Viruses 484.2.2009 and 367.2.2005 presented an insertion of 6 nt within the T4 variable region [44], corresponding to 341 amino acids capsid protein. The deduced lengths of RNA3 5'-UTRs and 3'-UTRs for the four betanodavirus strains spanned from 23 to 26 nt and from 122 to 124 nt respectively. RNA3 complete ORFs was 228 nt long, encoding 76 aa B2 protein.

The phylogenetic analysis based on the complete ORFs of RNA1 and RNA2 genetic segments confirmed that the 283.2009 and the 484.2.2009 isolates are indeed RGNNV-type and SJNNV-type respectively, and corroborated the identification of the RGNNV/SJNNV and SJNNV/RGNNV reassortants (samples 367.2.2005 and 389/I96, respectively) (Figure 1).

Nucleotide and amino acid similarities calculated for the RNA1 and the viral polymerase are 96.4% and 98% between strains 283.2009 and 367.2.2005, and 97.2% and 98.3% between strains 484.2.2009 and 389/I96. The RNA1 nucleotide sequence of the RGNNV and the RGNNV/SJNNV viruses was 97.6 and 96.7% identical to that of strain SGWak97 (corresponding to 99.3 and 97.6% amino acid identity). The polymerase gene of the SJNNV and of the SJNNV/RGNNV viruses was 97.1 and 97.8% identical to that of strain SJNag93 (corresponding to 97.8 and 98.1% amino acid identity). Pairwise similarity was calculated also for the RNA1 nucleotide region spanning from position 84 to position 1419 and putatively responsible for betanodavirus temperature sensitivity [35], which corresponds to the polymerase N-terminal. Nucleotide identity was 96.1% between strains 283.2009 and 367.2.2005 (corresponding to 98.4% amino acid identity) and 97.7% between strains 484.2.2009 and 389/I96 (corresponding to 97.7% amino acid identity). When considering the 84–1419 nucleotide region, the SGWak97 strain was 97.3 and 96.7% identical to samples 283.2009 and 367.2.2005 (100 and 98.4% amino acid identity, respectively), and the SJNag93 strain was 97.2 and 97.8% identical to samples 484.2.2009 and 389/I96 (96.8 and 97.9% amino acid identity, respectively). Finally, nucleotide and amino acid identities calculated for the RNA2 and the coat protein were 88.1% and 89.2% between strains 283.2009 and 389/I96, and 97.3% and 97% between strains 484.2.2009 and 367.2.2005. The SGWak97 strain was 97 and 88.1% identical to samples 283.2009 and 389/I96 (96.3 and 87.9% amino acid identity, respectively), and the SJNag93 strain was 97.8 and 99.1% identical to samples 484.2.2009 and 367.2.2005 (97 and 98.8% amino acid identity, respectively).

Phenotypic characterization

Inoculated cells were inspected regularly for the appearance of CPE (Table 2). At 15 °C, E-11 monolayers were characterized by cell shrinkage, most likely due to the suboptimal incubation temperature for their cultivation. From 93 hpi (T_7) onwards, an alteration of all the infected monolayers was observable, where cells appeared dark and contracted and tended to detach from the surface of the flask. However, no specific lesions related to betanodavirus infection (i.e. vacuolization of cells) were detected and none of the four viruses determined the complete disruption of cell monolayers at this temperature. At 20 °C, no CPE was noticed at the early stages of the infection. However, at 69 hpi (T_5) all the betanodavirus strains under investigation showed typical cellular vacuoles

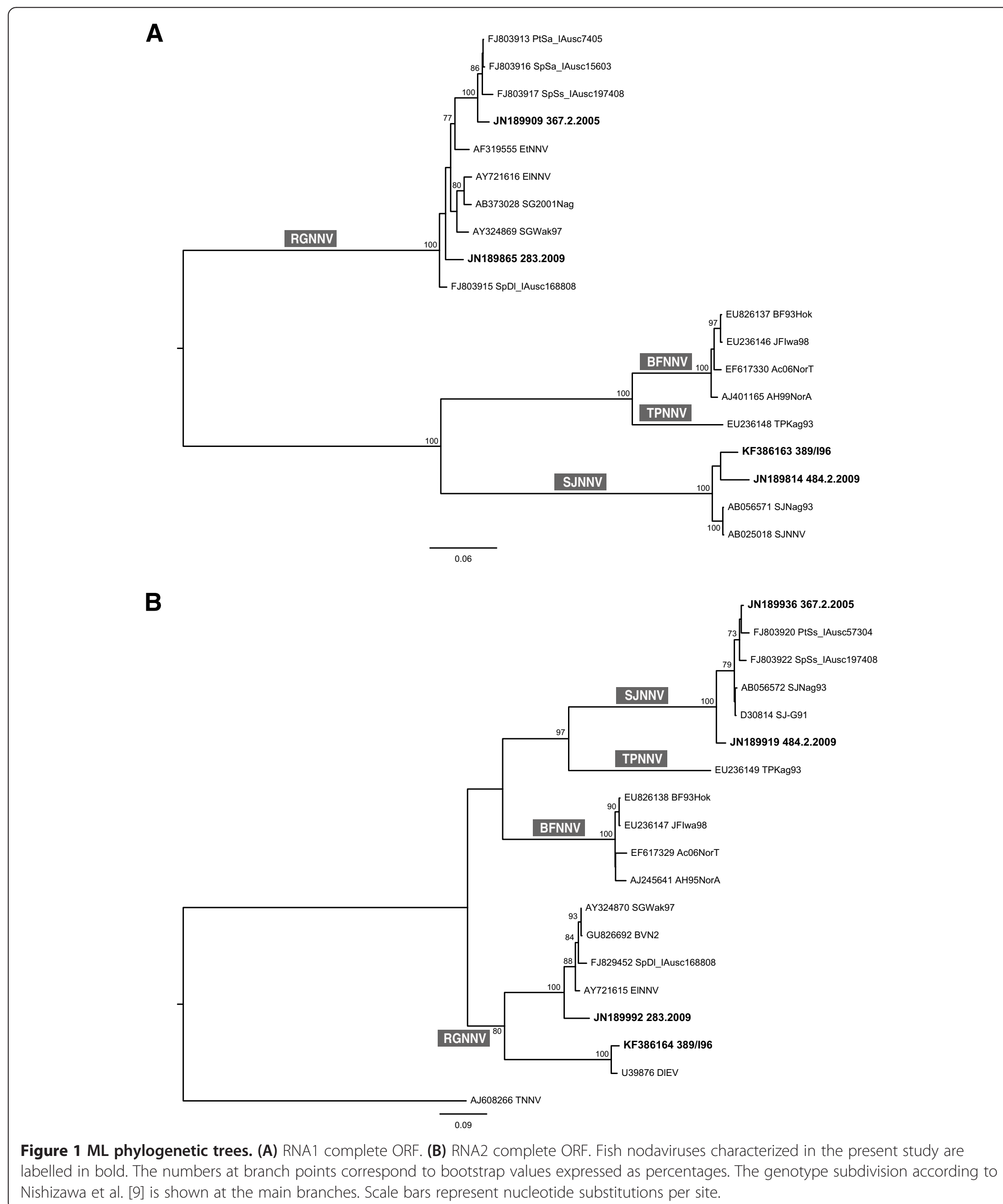

Figure 1 ML phylogenetic trees. (A) RNA1 complete ORF. **(B)** RNA2 complete ORF. Fish nodaviruses characterized in the present study are labelled in bold. The numbers at branch points correspond to bootstrap values expressed as percentages. The genotype subdivision according to Nishizawa et al. [9] is shown at the main branches. Scale bars represent nucleotide substitutions per site.

which evolved into extended *foci* of rounded, granular and vacuolated cells after 93 hpi (T_7). All the viral strains determined the complete disruption of cell monolayers by 117 hpi (T_8). At 25 °C, the RGNNV-type, the SJNNV-type and the two reassortants showed the same phenotype: after 69 hpi (T_5) the presence of diffuse CPE was observed, resulting in the disruption of the monolayers at 93 hpi (T_7). Interestingly, clear phenotypic differences among the

Table 2 Cytopathic effect (CPE) observed in E-11 cell monolayers infected with RGNNV, SJNNV, RGNNV/SJNNV, SJNNV/RGNNV betanodavirus strains at different incubation temperatures

	15 °C				20 °C				25 °C				30 °C			
	RGNNV	SJNNV	RGNNV/ SJNNV	SJNNV/ RGNNV	RGNNV	SJNNV	RGNNV/ SJNNV	SJNNV/ RGNNV	RGNNV	SJNNV	RGNNV/ SJNNV	SJNNV/ RGNNV	RGNNV	SJNNV	RGNNV/ SJNNV	SJNNV/ RGNNV
T1	§	§	§	§	-	-	-	-	-	-	-	-	-	-	-	-
T3	§	§	§	§	-	-	-	-	-	-	-	-	+ + +	-	+ + +	-
T5	§	§	§	§	+	+	+	+	+ +	+ +	+ +	+ +	+ + + +	-	+ + + +	+
T7	*	*	*	*	+ + + +	+ + + +	+ + + +	+ + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ +	+ + + + +	+ +
T8	*	*	*	*	+ + + + +	+ + + + +	+ + + + +	+ + + + +						+ + +		+ + +
T9	*	*	*	*										+ + + +		+ + + +
T10	*	*	*	*										+ + + +		+ + + +

Data were recorded at T_1 (20 hpi), T_3 (45 hpi), T_5 (69 hpi), T_7 (93 hpi), T_8 (117 hpi), T_9 (141 hpi) and T_{10} (165 hpi). Cells appearance, estimation of the CPE and its degree of severity were assessed according to the following levels: cell shrinkage (§); alteration of the cell monolayer characterized by dark and contracted cells with the tendency to detach from the surface of the flask (*); absence of CPE (–); cellular alteration and presence of diffuse vacuoles (+); presence of diffuse *foci* of rounded, granular and vacuolated cells (+ +); presence of diffuse *foci* of rounded, granular and vacuolated cells and initial cell monolayer disruption (+ + +); presence of diffuse *foci* of rounded, granular and vacuolated cells and advanced cell monolayer disruption (+ + + +); complete cell monolayer disruption (+ + + + +).

four betanodavirus strains were noticed at 30 °C. In particular, the RGNNV strain and the RGNNV/SJNNV reassortant determined an early appearance of CPE just after 45 hpi (T_3), which became more severe at 69 hpi (T_5). The complete disruption of cell monolayer was observable after 93 hpi (T_7). Noteworthy, the SJNNV strain and the SJNNV/RGNNV reassortant showed a completely different phenotype. In particular, the SJNNV/RGNNV strain determined the appearance of diffuse vacuoles after 69 hpi (T_5), while no CPE was observable for the SJNNV isolate. By 93 hpi (T_7) both the SJNNV strain and the SJNNV/RGNNV reassortant induced the emergence of multiple *foci* of vacuolated cells. CPE severity worsened over time, but none of these strains determined the complete disruption of the cell monolayers by the end of the experiment.

Growth kinetics

The average titres determined in culture supernatant samples collected at different time points were used to develop graphs describing the growth kinetics of the RGNNV and the SJNNV genotypes and their natural reassortants at different incubation temperatures (Figure 2). At 15 °C, a similar growth was noted for all the viruses, with a slight and slow increase of viral titre over time, although strains SJNNV and SJNNV/RGNNV seemed to have a better fitness, particularly towards the end of the experiment. In general, the chronic trend of viral growth indicates that this temperature is suboptimal for betanodavirus replication. The higher titres were recorded after 165 hpi (RGNNV: $10^{5.1}$ $TCID_{50}$/mL; SJNNV: $10^{5.7}$ $TCID_{50}$/mL; RGNNV/SJNNV: $10^{5.3}$ $TCID_{50}$/mL; SJNNV/RGNNV: $10^{6.1}$ $TCID_{50}$/mL). At 20 °C, all the viruses displayed comparable kinetics. However, this temperature showed a higher compatibility with an improved replication fitness, determining a regular increase of viral titre over time, particularly for strains SJNNV and SJNNV/RGNNV. The higher titres were obtained at 117 hpi (RGNNV: $10^{6.9}$ $TCID_{50}$/mL; SJNNV: $10^{6.8}$ $TCID_{50}$/mL; RGNNV/SJNNV: $10^{7.0}$ $TCID_{50}$/mL; SJNNV/RGNNV: $10^{7.4}$ $TCID_{50}$/mL). At 25 °C, the RGNNV strain and the two reassortants showed an acute growth trend characterized by a rapid and efficient replication, yielding high titres ($10^{7.9}$ $TCID_{50}$/mL) after 93 hpi. However, this condition was suboptimal for the SJNNV strain, which showed a slow growth and had a lower titre ($10^{6.3}$ $TCID_{50}$/mL after 93 hpi). Interestingly, at 30 °C the differences in replication efficiency among strains became striking. RGNNV and RGNNV/SJNNV rapidly multiplied during the first hours of incubation, reaching the peak of replication ($10^{7.5}$ $TCID_{50}$/mL and $10^{7.3}$ $TCID_{50}$/mL, respectively) only after 55 hpi. The acute phase was followed by a progressive decrease in viral titres, until the cell monolayer was completely disrupted. On the contrary, the SJNNV and the SJNNV/RGNNV betanodaviruses showed a chronic growth trend, characterized by a slow and poor replication. The higher titres were obtained at 117 hpi (SJNNV: $10^{5.5}$ $TCID_{50}$/mL; SJNNV/RGNNV: $10^{6.2}$ $TCID_{50}$/mL).

Statistical analysis

Given the diverse phenotypes and growth kinetics of viral strains at different incubation temperatures, data related to the observation points T_9 (141 hpi) and T_{10} (165 hpi) at 20 °C and T_8 (117 hpi), T_9 (141 hpi) and T_{10} (165 hpi) at 25 °C are missing for all viruses, as well as data associated with T_8 (117 hpi), T_9 (141 hpi) and T_{10} (165 hpi) at 30 °C for viruses RGNNV and RGNNV/SJNNV. This is why statistical analysis was performed using a reduced dataset, so as to have the same number of exposure times (up to T_7, corresponding to 93 hpi) for each temperature and genotype.

The first tested model, where replicates were considered as fixed effects, demonstrated the repeatability of the experiment, highlighting that differences in titres measurements among different replicates are not significant.

The best LMM selected for longitudinal data presented genotype, exposure time, temperature and their interaction as fixed effects while the replicate was considered as a random effect, with a AR(1) structure of VAR/COV matrix ($\rho = 0.18$). Graphical Student residuals analysis, Shapiro Wilks and Kolmogorov Smirnov tests of posterior analysis, confirmed the goodness of the proposed model (Additional file 1).

The analysis indicated that variables genotype, exposure time, temperature and their interactions were significant to explain the trend of the titres and highlighted the existence of differences among the increase of viral titres over time, depending on the temperature. In detail, the analysis showed that at 15 °C viral titres of strains RGNNV, SJNNV and SJNNV/RGNNV increased significantly more than RGNNV/SJNNV titres over time. Furthermore, no difference existed between the replication efficiency of pairs RGNNV and SJNNV, RGNNV and SJNNV/RGNNV, SJNNV and SJNNV/RGNNV over time. At 20 °C, no significant difference was observed between the increase of viral titres of strains RGNNV and RGNNV/SJNNV, and strains SJNNV and SJNNV/RGNNV over time, while isolates SJNNV and SJNNV/RGNNV showed a significantly higher replication efficiency when compared to strain RGNNV and strain RGNNV/SJNNV. At 25 °C and 30 °C, SJNNV viral titres increased significantly less than RGNNV, RGNNV/SJNNV and SJNNV/RGNNV titres over time. However, at both temperatures no significant differences were noticeable among the replication efficiencies of viruses RGNNV, RGNNV/SJNNV and SJNNV/RGNNV.

In order to evaluate whether the statistical observations might have been biased by the use of a restricted dataset, additional analyses were performed considering

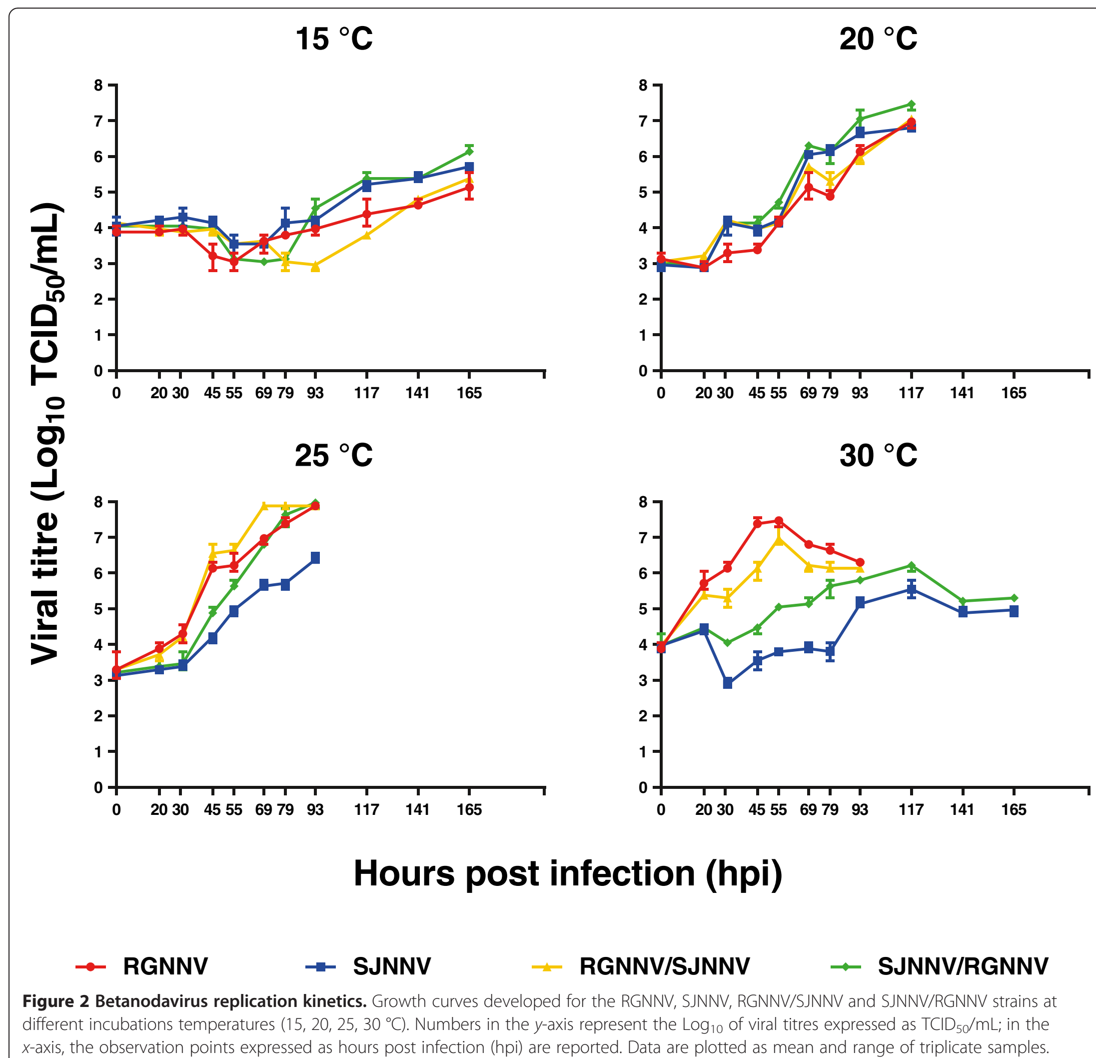

Figure 2 Betanodavirus replication kinetics. Growth curves developed for the RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV strains at different incubations temperatures (15, 20, 25, 30 °C). Numbers in the *y*-axis represent the Log_{10} of viral titres expressed as $TCID_{50}$/mL; in the *x*-axis, the observation points expressed as hours post infection (hpi) are reported. Data are plotted as mean and range of triplicate samples.

all the observation points up to 165 hpi for strains RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV at 15 °C, and for viruses SJNNV and SJNNV/RGNNV at 30 °C. The model developed for 15 °C showed no significant differences in the replication fitness of strains RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV. At 30 °C, the difference in terms of replication efficiency observed between strains SJNNV and SJNNV/RGNNV in the time slot T_0-T_7 was not significant when considering the entire duration of the experiment (up to T_{10} corresponding to 165 hpi).

Generally speaking, strains RGNNV and RGNNV/SJNNV showed similar growth trends at 15, 20, 25 and 30 °C. Similarly, the SJNNV and the SJNNV/RGNNV viruses exhibited comparable replication fitness at all the temperatures considered. Differences in viral titres between pairs RGNNV-RGNNV/SJNNV and SJNNV-SJNNV/RGNNV became more evident at 30 °C.

Discussion

Betanodavirus natural infections can occur at different water temperatures, depending on the genotype. The association between the onset of VNN and environmental conditions has been documented in several papers [45,46]. A number of experimental trials have also demonstrated the effect of temperature, infectious dose and

viral multiplication rate on betanodavirus pathogenicity and disease course [36,47-49]. These observations have led to the assumption that betanodavirus replication is most likely a temperature-sensitive process, as previously hypothesized also by Hata et al. [35]. With the aim of shedding light into the complex interplay existing between betanodavirus genetic features, environmental conditions and viral replication capacity, the present study investigates the effect of temperature on the in vitro replication of naïve RGNNV and SJNNV strains and on natural reassortants. We observed that all the viruses barely grew at 15 °C, while the rise of the incubation temperature up to 20 and 25 °C resulted in a boost of their multiplication capacity. The only exception was strain SJNNV, which showed a reduced fitness at 25 °C. A sharp increase in viral titre of the RGNNV and the RGNNV/SJNNV strains was noticeable at 30 °C, while the SJNNV and the SJNNV/RGNNV viruses showed a suboptimal growth kinetics comparable to that observed at 15 °C. Remarkably, viruses possessing the polymerase gene of the same genotype exhibited comparable replication trends, and strains with the RNA1 segment of the RGNNV genotype efficiently multiplied at higher temperatures (30 °C). All these data confirm that the RNA1 genetic segment and its encoded protein play a major role in controlling temperature sensitivity of fish nodaviruses, substantiating previous findings by Hata et al. [35]. Nevertheless, a possible role of the RNA2 and the RNA3 cannot be ruled out a priori although, to the best of the authors' knowledge, there is no evidence for the involvement of these molecules in the regulation of betanodavirus replication.

Interesting observations resulted from the sequencing analysis carried out in this study. Within the amino acid region 1–445 which controls betanodavirus temperature dependency [35], four transmembrane domains (TMDs) with moderate level of hydrophobicity were previously identified for the Greasy Grouper Nervous Necrosis Virus (GGNNV, belonging to the RGNNV genotype) [50] and the Atlantic Halibut Nodavirus (AHNV, grouping within the BFNNV genotype) [51]. These TMDs are located at positions 1–40, 225–246 for the AHNV, and at positions 153–173, 229–249 for the GGNNV. All but the TMD 153–173 were confirmed to contain mitochondrial targeting signals, responsible for the localization of protein A within the mitochondria membrane and for the formation of the replication complex. Interestingly, three putative TMDs located at positions 6–26, 152–173 and 224–249 were identified for the RGNNV, SJNNV, RGNNV/SJNNV and the SJNNV/RGNNV viruses herein characterized. TMD 224–249 was not predicted for strain SJNNV (data not shown), which notably replicates less efficiently at 25 and 30 °C. Furthermore, when comparing the RGNNV, SJNNV, RGNNV/SJNNV, SJNNV/RGNNV, SGWak97 and SJNag93 sequences corresponding to the TMDs identified by Guo et al. and Mézeth et al. [50,51], 9 amino acid signatures characteristic for each genotype were identified at positions 7, 19, 155, 223, 232, 235, 241, 251 and 254. In detail, 4 out of 9 signatures determined a dramatic change in the physical-chemical properties of the amino acids: $Ala_{7\text{-}RGNNV}$ vs $Glu_{7\text{-}SJNNV}$; $Met_{223\text{-}RGNNV}$/$Leu_{223\text{-}RGNNV}$ vs $Lys_{223\text{-}SJNNV}$; $Thr_{241\text{-}RGNNV}$ vs $Leu_{241\text{-}SJNNV}$; $Pro_{251\text{-}RGNNV}$ vs $Gln_{251\text{-}SJNNV}$. Positive-strand RNA viruses commonly associate their polymerase and viral RNA to the membranes of cellular organelles to replicate their genomes [52]. Several members of the *nodaviridae* family, namely AHNV, Flock house virus (FHV), GGNNV, Nodamura virus, Wuhan Nodavirus (WhNV), have shown to replicate in the mitochondria [50,51,53-55]. It is reasonable to speculate that the four betanodavirus strains herein described possess the same replication strategy, while the existence of specific amino acid signatures might relate with differences in growth kinetics of betanodaviruses with diverse genomes. The role of these mutations in determining protein A localization, membrane affinity, viral RNA recruitment, stability and accumulation as well as protein A interaction with host cell proteins is an issue which certainly deserves further investigations.

Results on viral replication obtained in this study were fully corroborated by extensive statistical analysis, and substantiate previous findings reported by Hata et al. [35], achieved by using the reverse genetics technique. Importantly, despite the methodological limits of our study due to the use of naïve reassortant strains, which have shown a certain degree of genetic diversity if compared to the parental RGNNV and SJNNV, the study of natural viruses give the most truthful picture of betanodavirus phenotype in response to temperature and definitively clarify the effect of genetic reassortment on viral replication. Interestingly, in our previous work we discovered that the polymerase gene evolves more rapidly than the viral capsid gene [23]. Whether this is a consequence of betanodavirus adaptive mechanisms to different climate and environmental conditions mainly regulated by RNA1, it is still an open question.

Recently, Vendramin et al. [36] have compared the pathogenicity of ten different betanodavirus strains, including samples 283.2009 (RGNNV), 484.2.2009 (SJNNV), 367.2.2005 (RGNNV/SJNNV) and 389/I96 (SJNNV/RGNNV). Sea bass juveniles were bath challenged at 20 °C, and were subsequently subjected to gradually increasing water temperatures (23 and 25 °C). Overall, it was observed that the mortality rate proportionally increased with water temperature. Noteworthy, strain 283.2009 (RGNNV) was the most lethal virus, reassortant strains 389/I96 and 367.2.2005 increased their pathogenicity at 23 and 25 °C respectively, and virus 484.2.2009 (SJNNV) was lethal for

fish at 20 °C without any further increase in pathogenicity at higher temperatures. Generally speaking, the in vivo results obtained by Vendramin et al. are in agreement with the outcomes of the present study, and the mortality rate determined by different betanodavirus strains seems to reflect their in vitro multiplication capacity at 20 and 25 °C, suggesting that replication efficiency is crucial for betanodavirus pathogenicity. However, our results indicate that although both the SJNNV/RGNNV and the SJNNV strains induced CPE at 30 °C but not at 15 °C, each virus reached nearly identical titres when the two incubation temperatures were compared. This means that for the SJNNV/RGNNV and the SJNNV genotypes the appearance of CPE is not exclusively dose-dependent, which suggests that temperature might influence viral phenotype through different mechanisms other than replication efficiency.

Data gained in the present study might have practical implications, as they could help infer the viral phenotype on the basis of the genetic data. Furthermore, the in vitro characterization of the viral phenotype appears to be a suitable methodology for the prediction of betanodavirus pathogenicity under controlled environmental conditions, and might have important applications in fish farming and vaccinology. Indeed, Nishizawa et al. [49] applied the principle of regulating viral pathogenicity by controlling fish rearing temperature, and immunized sevenband grouper (*Epinephelus septemfasciatus*) with a live vaccine strain by keeping the water temperature at 17 °C. This vaccination strategy, despite the obvious limitations for application in open farms or sea cages, might be used inside hatcheries or, alternatively, during transportation of fish to reduce the volume of vaccine needed [56]. Additionally, the identification of viruses which replicate more efficiently in vitro may assist in selecting a candidate vaccine strain suitable for high-throughput antigen production.

Additional file

Additional file 1: Graph Student residual analysis: residuals versus predicted plot, residuals distribution and residuals Q-Q plot. Residuals without a particular trend, with normal distribution and good alignment over line indicate that the model is correct. The hypothesis of normal distribution of residuals is further tested using the Shapiro-Wilk and Kolmogorov-Smirnov test. Value of $p > 0.10$ indicates that the residual has a normal distribution.

Abbreviations

VNN: viral nervous necrosis; VER: viral encephalopathy and retinopathy; RdRp: RNA-dependant RNA polymerase; CP: coat protein; SJNNV: Striped jack nervous necrosis virus; TPNNV: Tiger puffer nervous necrosis virus; BFNNV: Barfin flounder nervous necrosis virus; RGNNV: Red-spotted grouper nervous necrosis virus; TNNV: Turbot nervous necrosis virus; SSN-1: Striped snakehead cell line; FCS: Foetal calf serum; CPE: Cytopathic effect; TAP: Tobacco acid pyrophosphatase; DMSO: Dimethyl sulfoxide; RNA: Ribonucleic acid; RT: Reverse transcription; cDNA: Complementary deoxyribonucleic acid; PCR: Polymerase chain reaction; dNTPs: Deoxinucleotides; ML: Maximum likelihood; GTR: General time-reversible; MOI: Multiplicity of infection; TCID: Tissue culture infectious dose; LMM: Linear mixed model; VAR/COV: Variance and covariance matrix; CS: Compound symmetry; CSH: Heterogeneous compound symmetry; UN: Unstructured, AR(1): first order autoregressive; AR(1): First order autoregressive; UTRs: Untranslated regions; ORF: Open reading frame; TMD: Transmembrane domain; GGNV: Greasy grouper nervous necrosis virus; AHNV: Atlantic halibut nodavirus; FHV: Flock house virus; WhNV: Wuhan nodavirus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GC conceived the study and coordinated the work described. VP, AT, CT were involved in the experimental design. EC, VP, AM performed the experiments and interpreted the results. MM carried out the statistical analysis. VP wrote the manuscript. GC, AT, CT were involved in the interpretation of the results and critically read the manuscript. All authors read and approved the final manuscript.

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Chapter 8

Comparative pathogenicity study of ten different betanodavirus strains in experimentally infected European sea bass, *Dicentrarchus labrax*(L.)

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Comparative pathogenicity study of ten different betanodavirus strains in experimentally infected European sea bass, *Dicentrarchus labrax* (L.)

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Abstract

Viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), is a severe pathological condition caused by RNA viruses belonging to the *Nodaviridae* family, genus *Betanodavirus*. The disease, described in more than 50 fish species worldwide, is considered as the most serious viral threat affecting marine farmed species in the Mediterranean region, thus representing one of the bottlenecks for further development of the aquaculture industry. To date, four different genotypes have been identified, namely red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus and barfin flounder nervous necrosis virus, with the RGNNV genotype appearing as the most widespread in the Mediterranean region, although SJNNV-type strains and reassortant viruses have also been reported. The existence of these genetically different strains could be the reason for the differences in mortality observed in the field. However, very little experimental data are available on the pathogenicity of these viruses in farmed fish. Therefore, in this study, the pathogenicity of 10 isolates has been assessed with an *in vivo* trial. The investigation was conducted using the European sea bass, the first target fish species for the disease in the Mediterranean basin. Naive fish were challenged by immersion and clinical signs and mortality were recorded for 68 days; furthermore, samples collected at selected time points were analysed to evaluate the development of the infection. Finally, survivors were weighed to estimate the growth reduction. The statistically supported results obtained in this study demonstrated different pathogenicity patterns, underlined the potential risk represented by different strains in the transmission of the infection to highly susceptible species and highlighted the indirect damage caused by a clinical outbreak of VER/VNN.

Keywords: betanodavirus, European sea bass, pathogenicity, viral encephalopathy and retinopathy.

[Correction added on 2 August 2013, after first online publication: The strain DI-I-96 was amended to 389/I96. Figures 1, 2, 3, 6, 7 and Tables 2, 3 have been updated to reflect the change.]

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Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), has been detected in the Mediterranean area since 1991 (Breuil *et al.* 1991), becoming a serious problem for aquaculture development.

The aetiological agent of VER/VNN belongs to the family *Nodaviridae*, genus *Betanodavirus*; it is an icosahedral, non-enveloped viral particle of about 25 nm in diameter, with a bisegmented genome composed of two single-stranded positive-sense RNA molecules. The RNA1 segment (3.1 Kb) encodes the

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RNA-dependent RNA polymerase (RdRp) or protein A, while the RNA2 segment (1.4 Kb) encodes the coat protein (Mori *et al.* 1992). The RNA3 subgenomic transcript (0.4 Kb) is synthesized from the 3′ terminus of RNA1 molecule during RNA replication and encodes protein B1 and B2, which act as an antinecrotic cell death function and antagonize host cell RNA interference mechanisms, respectively (Fenner *et al.* 2006; Chen, Su & Hong 2009).

According to the phylogenetic analysis of the T4 variable region within the RNA2 segment, betanodaviruses can be classified into four different genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.* 1997). More recently, genetic analysis based on both RNA1 and RNA2 has demonstrated the existence of reassortant viruses (RGNNV/SJNNV and SJNNV/RGNNV) (Toffolo *et al.* 2007; Olveira *et al.* 2009; Panzarin *et al.* 2012). Because of their genetic differences, two additional betanodavirus genotypes have been proposed: the Atlantic cod nervous necrosis virus (Gagné *et al.* 2004) and the turbot nodavirus (Johansen *et al.* 2004).

The disease is primarily transmitted horizontally, although vertical transmission is strongly suspected to occur in some species (Breuil *et al.* 2002).

European sea bass, *Dicentrarchus labrax* (L.), is considered the main target species in the Mediterranean aquaculture industry. The losses in the field can vary from 11 to 60% in sea cages and from 11 to 50% in tanks (Le Breton *et al.* 1997; Chérif *et al.* 2009): different hypotheses have been suggested to explain the different mortality rate, such as the size/age of the affected fish and environmental conditions (i.e. water temperature). It is already known that different VER/VNN genotypes have diverse optimal *in vitro* growth temperatures (Iwamoto *et al.* 2000; Hata *et al.* 2010), and these differences seem to correspond to a changed *in vivo* pathogenicity (Table 1). Strain pathogenicity has also been considered: under experimental conditions two different viral strains revealed different pathogenicity in European sea bass larvae (Breuil *et al.* 2001).

Table 1 Optimum growth temperature RGNNV genotype and SJNNV genotype (OIE Manual of Diagnostic Tests for Aquatic Animals 2012, Chapter 2.3.11)

Genotype	Target host fish	Optimum growth temperature (°C)
SJNNV	Striped jack	20–25
RGNNV	Warm-water fish: Asian sea bass, European sea bass, groupers, etc.	25–30

SJNNV, striped jack nervous necrosis virus; RGNNV, red-spotted grouper nervous necrosis virus.

Fish affected by VER/VNN generally show clear clinical signs, such as anorexia, skin darkening, blindness and abnormal swimming behaviour. Typical histological lesions include cellular vacuolation, necrosis and neuronal degeneration in the central nervous system (CNS).

Two different clinical forms of VER/VNN were suggested by Sweetman *et al.* (1996): (i) the acute form, characterized by a higher mortality rate and the classical nervous form and (ii) a subacute form, characterized by lower mortality and nonspecific clinical signs.

In addition to European sea bass, other species reared in the same environment are known to be severely affected by betanodavirus, such as: shi drum, *Umbrina cirrosa* (L.), sharp snout sea bream, *Diplodus puntazzo* (Walbaum), brown meagre, *Sciaena umbra* (L.), meagre, *Argyrosomus regius* (Asso), and Senegalese sole, *Solea senegalensis* (Kaup) (Munday, Kwang & Moody 2002). The gilthead sea bream, *Sparus aurata* (L.), together with the sea bass, represents the most important farmed species in the Mediterranean and has always been considered resistant to the disease/virus, due to the lack of clinical signs and mortality, even if infected and farmed close to sea bass suffering from serious mortality rates. However, severe outbreaks in sea bream, inducing mass mortalities during larval stages, have been recently reported (Beraldo *et al.* 2011).

In the Mediterranean basin, different genotypes have been detected; however, the RGNNV genotype appears to be the most common, showing the broadest host range and causing infection in a variety of warm-water fish species (Olveira *et al.* 2009). The SJNNV genotype, initially believed to be limited to a few species present in Japanese waters (Munday *et al.* 2002), has recently affected some marine species reared in the Mediterranean area, such as the Senegalese sole (Thiéry *et al.* 2004; Panzarin *et al.* 2012), but strains belonging to this parental genotype had not until now been detected in European sea bass. Finally, reassortant RGNNV/SJNNV strains have been responsible for clinical outbreaks of the disease in this area (Toffolo *et al.* 2007; Olveira *et al.* 2009). To

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date, little is known about the pathogenicity of different betanodavirus genotypes, including the reassortant viruses, in fish species of the Mediterranean Sea.

Recently, data on the influence of water temperature in disease development with reassortant strains using the Senegalese sole as a model host for *in vivo* challenge have been produced; nevertheless, data referring to sea bass are lacking (Souto *et al.* 2010, 2011). Thus, to compare the pathogenicity of both parental and reassortant strains for European sea bass, a panel of ten different betanodavirus isolates was selected and tested in an experimental infection trial.

Materials and methods

Fish

A batch of 1000 sea bass juveniles (0.2 g mean weight) purchased from a commercial marine hatchery was introduced into aquarium facilities. To ascertain the VER/VNN status of the newly introduced fish, three different pools of samples consisting of 10 fish each were tested by real-time RT-PCR for betanodavirus (Panzarin *et al.* 2010) and cell culture isolation on SSN-1 cells (Frerichs, Rodger & Peric 1996).

Challenge viruses

A panel of ten betanodavirus strains was selected for the experimental challenges in sea bass (Table 2). From data made available in this study and previous investigations (Beraldo *et al.* 2011; Bovo *et al.* 2011; Panzarin *et al.* 2012), viruses were sorted according to their genotype, the host species from which they were isolated, namely *D. labrax* (L.), *S. aurata* L., *Micropterus salmoides* (Lacepède), *S. senegalensis* (Kaup) and the aquatic environment (i.e. marine or fresh water). Virus strain 378/I02-RGNNV was used as control virus because of its known pathogenicity under experimental conditions, as proved by previous experiments (approximately 30% mortality at 25 °C, in experimentally infected European sea bass, laboratory internal data). Betanodavirus isolates were propagated and titrated in E-11 cell monolayers (Iwamoto *et al.* 2000). Viral titres were expressed in $TCID_{50}$ mL^{-1} according to Reed & Muench (1938). Prior to the challenge, viral titres were normalized to $10^{8,3}$ $TCID_{50}$ mL^{-1} and then diluted

Table 2 Data on the fish betanodavirus isolates tested in this study

ID of the isolate	Species of origin	Aquatic environment	Clinical signs in the species of origin	Genotype (RNA1 and RNA2)	Back titration value of challenge bath ($TCID_{50}$/mL)	GenBank acc. no RNA1	GenBank acc. no RNA2	Reference
378/I02 laboratory reference isolate	*Dicentrarchus labrax*	SW	Present	RGNNV	$10^{4.30}$	JX290515	JX290517	This work
389/I96	*D. labrax*	SW	N.A.	SJNNV/RGNNV	$10^{4.05}$	KF386163	KF386164	This work
367-2/I05	*D. labrax*	SW	Present	RGNNV/SJNNV	$10^{4.30}$	JN189909	JN189936	Panzarin *et al.* (2012)
367-3/I05	*D. labrax*	SW	Present	RGNNV	$10^{4.30}$	JN189876	JN190000	Panzarin *et al.* (2012)
324/I06	*Sparus aurata*	SW	N.A.	RGNNV	$10^{4.05}$	JN189820	JN189972	Panzarin *et al.* (2012)
82/I07	*S. aurata*	SW	Present	RGNNV/SJNNV	$10^{4.05}$	JX290516	JX290518	Beraldo *et al.* (2011)
243/I09	*Micropterus salmoides*	FW	Present	RGNNV	$10^{4.30}$	JQ970429	JQ970432	Bovo *et al.* (2011)
250/I09	*S. aurata*	SW	N.A.	RGNNV/SJNNV	$10^{4.05}$	JN189815	JN189920	Panzarin *et al.* (2012)
283/I09	*D. labrax*	SW	Present	RGNNV	$10^{4.05}$	JN189865	JN189992	Panzarin *et al.* (2012)
484-2/I09	*Solea senegalensis*	SW	Absent	SJNNV	$10^{4.05}$	JN189814	JN189919	Panzarin *et al.* (2012)

N.A., not available; SW, salt water; FW, fresh water; SJNNV, striped jack nervous necrosis virus; RGNNV, red-spotted grouper nervous necrosis virus.

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in marine water to reach the infectious dose of 10^4 $TCID_{50}$ mL^{-1}. After 2 h water samples were collected from the bath challenge for back titration according to the same protocol.

RNA1 and RNA2 nucleotide sequences related to the challenge viruses were aligned and compared with reference sequences retrieved from GenBank using the MEGA 4 package (Tamura *et al.* 2007). For both the RNA1 and RNA2 segments, phylogenetic trees were developed using the neighbour-joining (NJ) method with 1000 bootstrap resamplings. Pairwise similarities were also determined.

Experimental design

Twenty days after their arrival, fish were randomly divided into 11 distinct experimental groups, each consisting of 80 fish. Infections were performed by immersing each group for 2 h in 10 L of artificial salt water (salinity 2.5%, water temperature 20 °C) containing the diluted infected cell supernatant previously titrated. The negative control group underwent a bath with non-infected artificial sea water for the same time and in the same conditions. After infection, water samples collected from each tank were back titrated.

Each group was then transferred to a 300 L fibreglass aquaria filled with the marine artificial water (2.5% salinity); external biological filters were used to stabilize water physicochemical parameters throughout the experimental period. Fish were fed daily with the same commercial diet used in the hatchery (LARVIVA ProWean, Biomar group).

At the beginning of the experiment, water temperature (T°C) was set at 20 °C. Thirty-three days post-infection (d.p.i.), T°C was slowly raised to 23 °C and finally, at 56 d.p.i., the temperature was raised again and fish gradually acclimatized to 25 °C until the end of the trial (day 68).

The water temperature was gradually raised to evaluate the viral strain pathogenicity under suitable conditions for disease development (Le Breton *et al.* 1997; Souto *et al.* 2010, 2011) aiming to mimic the environmental conditions that are commonly associated with clinical outbreaks in a Mediterranean farm.

Infected and uninfected groups were regularly monitored (at least twice a day) for the appearance of clinical signs and mortality. Dead and moribund fish were removed as soon as possible from the tanks and individually tested by real-time RT-PCR to assess the presence of the viral genome. At 68 days post-infection, survivors were harvested and 30 specimens randomly selected were individually weighed and stored at −20 °C until tested.

To evaluate the persistence of the infection, even in the absence of clinical signs, five apparently healthy fish were harvested from each experimental group at three selected time points: 8 d.p.i. (acute stage T°C = 20), 44 d.p.i. (intermediate stage T°C = 23) and 68 d.p.i. (chronic stage T°C = 25) and tested by cell culture isolation and real-time RT-PCR.

The RNA1 and RNA2 genome segments of the viruses isolated from each tank at the end point of the experiment were sequenced according to a previously published protocol (Panzarin *et al.* 2012), and the sequences obtained were compared with the original sequence of the virus used for the challenge to rule out potential cross-contamination during the trial.

Laboratory testing procedures

Virological investigations. Virus isolation from the nervous tissues was performed on the pools of five apparently healthy fish collected, respectively, at 8, 44 and 68 d.p.i. to ascertain the presence of the virus at these time points. Samples consisting of CNS were homogenized by mortar and pestle with sterile sand, suspended at a final dilution 1:10 in Eagle's minimum essential medium, supplemented with 10% v/v foetal calf serum and 2% v/v of antibiotic–antimycotic solution (penicillin 100 UI mL^{-1}, streptomycin sulphate 10 mg mL^{-1}, amphotericin B 25 μg mL^{-1} and kanamycin 10 mg mL^{-1}). Tissue homogenates were clarified by centrifugation at 3000 ***g*** for 15 min, and the supernatants collected were incubated overnight at 4 °C. According to internal quality assurance validated procedures, homogenate supernatants were inoculated at two- to tenfold dilutions (1:10 and 1:100) onto 1-day-old SSN-1 cells (Frerichs *et al.* 1996) grown in 24-well cell culture plates and incubated at 25 °C. After inoculation, plates were observed daily for cytopathic effect (cpe) detection. If no cpe was detected after 10 days, inoculated cultures were subcultured for further 10 days.

Real-time RT-PCR. Samples were processed by real-time RT-PCR according to a published

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protocol (Panzarin *et al.* 2010). The CNS of each dead fish was analysed individually, while CNS of apparently healthy fish sampled at selected time points were examined in pools of five specimens. Samples were homogenized by mortar and pestle with sterile sand and clarified by centrifugation at 3000 ***g*** for 15 min. Total RNA was extracted from 100 μL of tissue supernatant using the NucleoSpin® RNA II (Macherey-Nagel GmbH & Co.), according to the manufacturer's instructions. After cDNA synthesis with the 'High-Capacity cDNA Reverse Transcription Kit' (Applied Biosystem) using 15 μL total RNA per reaction, real-time PCR was conducted on the LightCycler 2.0 system using the 'LightCycler® TaqMan® Master' (Roche Diagnostics GmbH) following the supplier's recommendations. Primers and probes were used at a final concentration of 0.9 and 0.75 μM, respectively, while thermal cycling was performed at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 35 s and 72 °C for 1 s, with a final incubation at 40 °C for 30 s. Data analyses were performed with LightCycler software 4. The cut-off value was set at 36 CP (crossing point) based on results of internal validation.

Statistical analysis

The cumulative mortality percentage (Figs 4–7) and the survival rate (Fig. 2) were calculated without considering the number of fish withdrawn for sampling at selected time points (i.e. cumulative mortality percentage was calculated on the entire batch consisting of 80 fish).

To analyse the survival function from lifetime data, the Kaplan–Meier estimator was used. This estimator allows drawing the survival curve as a step curve for each group, measuring the length of time that fish survive the infection. In the graph, the *y*-axis plots the cumulative proportion of surviving fish and therefore it is possible to calculate the survival probability at every time point for each group (van Belle *et al.* 2004).

As the condition of proportional hazards was not verified for each virus (overlapping of curves), the semi-parametric Cox model, to identify differences in survival numbers, was not applicable. Therefore, the nonparametric log-rank test was applied to verify the equality of survivor functions across groups (van Belle *et al.* 2004).

Weights of survivors were analysed by descriptive statistical analysis providing the mean, median and standard deviation for each group (data not shown) and constructing box-and-whiskers plot to show data distribution.

An appropriate statistical test was conducted to verify whether a significant difference existed between the weight of the compared groups:

- If the hypothesis of normality and homoscedasticity of data were both satisfied, the parametric Student's *t*-test for two independent samples was used. This test compares the average weight of groups (van Belle *et al.* 2004).
- If the hypothesis of normality was not satisfied but the hypothesis of homoscedasticity of the variances between the compared groups was, the nonparametric Wilcoxon–Mann–Whitney test for two independent samples was conducted. This test also compares the distribution weight of groups (van Belle *et al.* 2004).
- If the hypothesis of normality and homoscedasticity were not satisfied, the nonparametric Smirnov test for two independent samples was used. This test also compares the distribution weight of groups (van Belle *et al.* 2004).

The Shapiro–Wilk test (Shapiro & Wilk 1965) was conducted to verify the normality of the weight distribution for each group. This test is the most powerful normality test and it is able to detect departures from normality due to either skewness or kurtosis, or both.

The *F* and the Levene's robust tests (Levene 1960) were performed to verify the equality of variance of each pair of groups according to the data distribution. The traditional *F* test for the homogeneity of variances relies on the assumption that data are drawn from an underlying Gaussian distribution, while Levene's test is found to be more robust under non-normality.

Results

Phylogenetic analysis of challenge VER/VNN strains

The challenge panel included $n = 5$ RGNNV, $n = 1$ SJNNV, $n = 3$ RGNNV/SJNNV and $n = 1$ SJNNV/RGNNV strains. The phylogenetic analysis is shown in Fig. 1a, b. Nucleotide similarity calculated among the challenge viruses ranged from 99.9 to 74.6% for RNA1 and from 99.8 to 62.5% for

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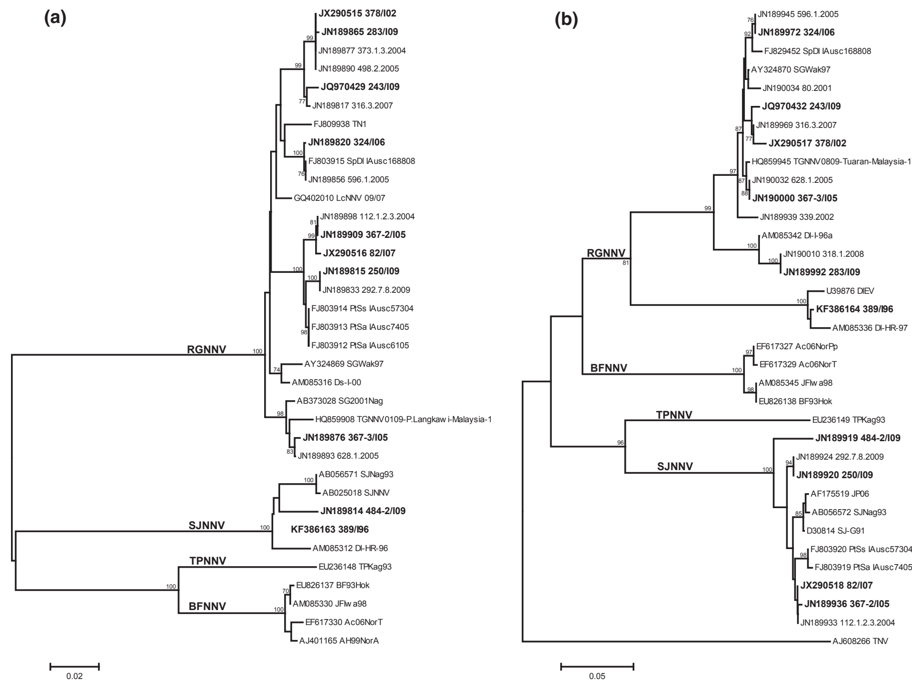

Figure 1 RNA1 (a) and RNA2 (b) phylogenetic trees inferred using the neighbour-joining (NJ) method. Isolates used for experimental infections are labelled in bold. Challenge viruses are compared to betanodavirus representative sequences retrieved from GenBank. The numbers at branch points represent bootstrap values expressed as percentages. The genotype subdivision according to Nishizawa *et al.* (1997) is shown at the main branches. Scale bar represents the number of substitution per site.

RNA2, while amino acid identity ranged from 100 to 88.9% for RNA1 and from 100 to 78.6% for RNA2.

Clinical signs, statistical analysis and mortality rate

No significant mortality was recorded during the quarantine period, and virological analysis carried out both on cell culture and with real-time RT-PCR during the quarantine period of 20 days provided negative results, confirming the VER/VNN free status of the sea bass batch used for the challenge trial.

While no clinical signs were observed in the control group, typical signs appeared 9–10 d.p.i. with slight differences in all the infected groups. The affected fish showed an abnormal swimming behaviour characterized by whirling, surfing and ataxia, skin darkening and mortality. The acute clinical phase characterized by such signs lasted approximately 20 days for each tank, then the situation normalized although the fish continued to display a certain degree of lethargy and non-active feeding response in all the infected groups; in all the infected tanks, it was possible to detect the presence of fish suffering from starvation. In two experimental tanks (82/I07-RGNNV/SJNNV and 367-2/I05-RGNNV/SJNNV), mortality was raised after the end of the acute symptomatic phase. The Kaplan–Meier survival functions represented the pathogenicity of each compared group, so that the lower curve corresponded to the most lethal virus (283/I09-RGNNV) and the higher curve corresponded to the negative control group (Fig. 2). The nonparametric log-rank test applied to survival functions indicated that a statistically significant difference existed among all groups ($P < 0.01$). In particular, the pair comparison between each virus and control group showed a significant difference in the survival function ($P < 0.05$) (Fig. 2). These results highlighted the

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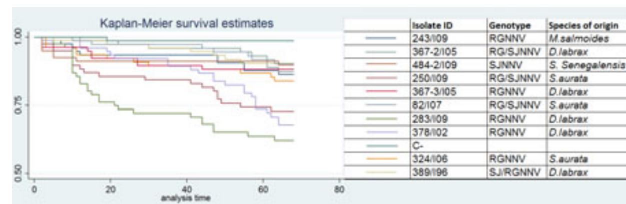

Figure 2 Kaplan–Meyer survival curves. The pathogenicity of each group is plotted so that the lower curve corresponded to the most lethal virus and the higher curve corresponds to the negative control group. Each step curve represents one experimental group measuring the length of time that fish survive the infection.

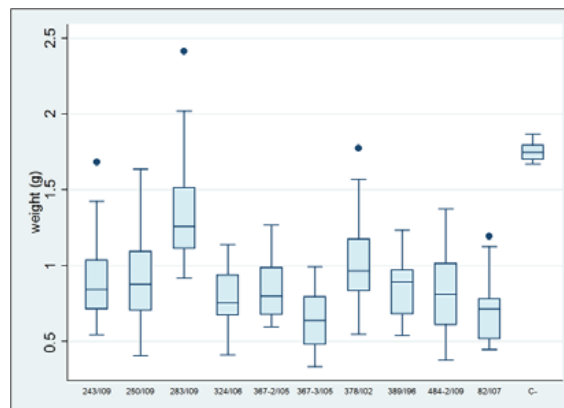

Figure 3 Box-and-whiskers plot of weight values, by virus. The box identifies the middle 50% of the results; the lower part of the box represents the first quartile, and the top part, the third quartile. The horizontal line inside the box is the median. The vertical lines protruding from the box extend to the minimum and the maximum values of the data set, as long as these values do not differ from the median by more than one and half times the interquartile range. The extremities of the whiskers are marked by two shorter horizontal lines. The values above the top whisker (marked with the blue point) are considered outliers.

statistically significant pathogenicity of each virus compared with the negative control group.

Descriptive analysis and box-and-whiskers plot analysis showed that in each experimental group, the unevenness in size, assessed by weight variability, was significantly higher for the infected fish compared with that observed in uninfected fish (Fig. 3). The analysis of homoscedasticity of variances indicates that all infected groups showed a significantly higher variability in weight than the control group ($P < 0.01$). Furthermore, the weight of the control group was significantly greater than the weight of all infected groups ($P < 0.01$). Among the infected fish, the group challenged with virus 283/I09-RGNNV weighed significantly more than all other infected groups ($P < 0.01$) although this was a reduction of approximately 35% in weight compared with the control group. The group infected with virus 367-3/I05-RGNNV showed significantly lower weights than all the other groups ($P < 0.01$) with the exception of the group infected with strain 82/I07-RGNNV/SJNNV in which the *P*-value was not significant. No differences were established among the groups infected with 324/I06-RGNNV, 367-3/I05-RGNNV and 484-2/I09-SJNNV. Comparisons are presented in Fig. 3.

The cumulative mortality observed during the experiment in the different groups following the viral challenge is shown in Figs 4–6 and summarized in Fig. 7.

Overall, mortality rate for a given strain increased with increasing water temperature; however, notable differences and exceptions were observed among the strains. The mortality rate for the 378/I02-RGNNV strain, included as positive control, was 27.5%, while the most pathogenic strain found in this study, 283/

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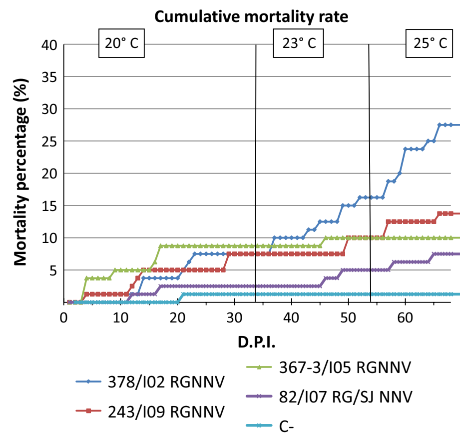

Figure 4 Cumulative mortality expressed in percentage (%) observed in groups infected with isolates 82/I07, 367-3/I05, 243/I09 and 378/I02 (laboratory ref. isolate). The negative control group is represented by the blue line.

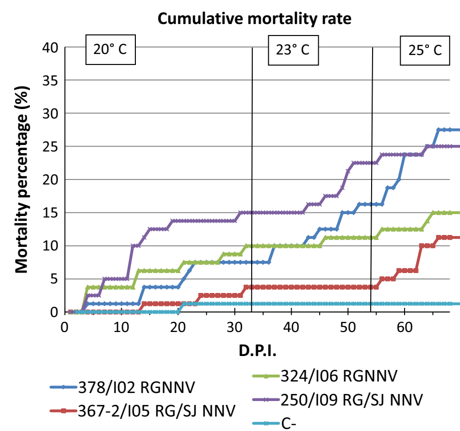

Figure 5 Cumulative mortality expressed in percentage (%) observed in groups infected with isolates 250/I09; 324/I06, 367-2/I05 and 378/I02 (laboratory ref. isolate). The negative control group is represented by the blue line.

I09-RGNNV, exhibited a mortality rate of 36.25%. The least pathogenic virus was a reassortant strain isolated from gilthead sea bream, strain 82/I07-RGNNV/SJNNV, exhibiting 7.5% cumulative mortality (Fig. 7). Interestingly, two of three virus strains, i.e. strain 283/I09–RGNNV and strain 250/I09–RGNNV/SJNNV (Figs 5 & 6), exhibiting the highest cumulative mortalities ($\geq$25%) at the end of the

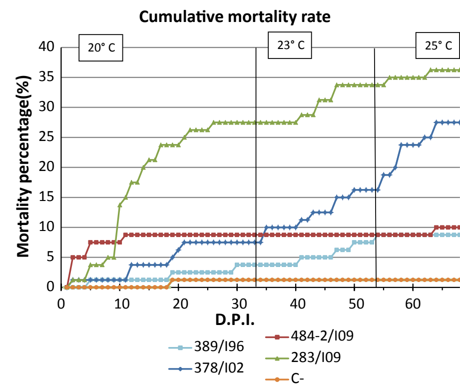

Figure 6 Cumulative mortality expressed in percentage (%) observed in groups infected with isolates 389/I96, 283/I09, 484-2/I09 and 378/I02 (laboratory ref. isolate). The negative control group is represented by the blue line.

experiment, caused a higher mortality rate in the earlier phase of the experiment, at 20 °C of water temperature. On the other hand, for the majority of the other strains (including 378/I02-RGNNV), the mortality curve increased with the increase of water temperature (Figs 4–6). Strains 367-3/I05-RGNNV and 484-2/I09-SJNNV revealed a high mortality rate at the beginning of the experiment, at 20 °C, which then stopped to the end of the trial. In fact, in group 484-2/I09-SJNNV, only 1 fish died after the raising of the temperature (from 23 to 25 °C) and this tested negative by real-time RT-PCR. In contrast, for strain 367-2/I05-RGNNV/SJNNV, the mortality rate appeared similar to that of the uninfected control group during the first 50 days of the infection; after this time span, it increased sharply at the end of the experiment, when T°C was raised from 23 to 25 °C (Fig. 5).

Virological investigations at selected time points and real-time RT-PCR on dead specimens

Virus isolation and real-time RT-PCR performed on a pool of five CNS from fish collected at 8, 44 and 68 d.p.i. always yielded positive results, except for samples collected from 484-2/I09-SJNNV-infected fish on day 68 p.i. The detailed results are reported in Table 3.

A total of 134 dead fish were collected, 110 of which were suitable for analysis and of these, 100 tested positive by real-time RT-PCR.

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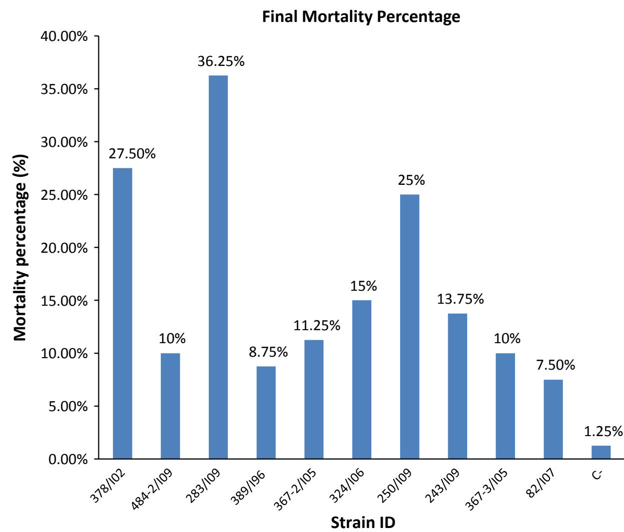

STRAIN ID	Genotype	Species of Origin
378/I02	RGNNV	*D.labrax* L.
484-2/I09	SJNNV	*S. Senegalensis* Kaup
283/I09	RGNNV	*D.labrax* L.
389/I96	SJ/RG NNV	*D.labrax* L.
367-2/I05	RG/SJ NNV	*D.labrax* L.
324/I06	RGNNV	*S.aurata* L.
250/I09	RG/SJ NNV	*S.aurata* L.
243/I09	RGNNV	*M.salmoides* Lacepède
367-3/I05	RGNNV	*D.labrax* L.
82/I07	RG/SJ NNV	*S.aurata* L.

Figure 7 Histogram representing cumulative mortality rate at the end of the trial (68 d.p.i.).

The remaining ten negative samples were dead fish collected from the negative control tank, to those randomly distributed in experimental tanks, collected within the second d.p.i. (probably died as a consequence of handling operations) and to one dead fish collected at day 64 p.i. from the tank infected with strain 484-2/I09-SJNNV.

Discussion

To the best of our knowledge, the pathogenicity of different betanodavirus strains in European sea bass has been compared only once. Experimental trials performed by Breuil *et al.* (2001) revealed that an Atlantic strain of nodavirus was more pathogenic for sea bass embryos than a Mediterranean one; furthermore, a difference in the kinetic of the mortality was observed. The trial revealed that mortality increased more rapidly in sea bass infected with the Atlantic strain. Recently, the pathogenicity of four different strains of betanodavirus, belonging to reassortant and parental genotypes, has also been investigated in Senegalese sole (Souto *et al.* 2010), and also, the mortality kinetics of a reassortant strain, i.e. SpSSIAusc 160.03 were investigated at different temperatures (Souto *et al.* 2011). This species was extremely susceptible to the disease, resulting in 100% mortality when temperature was raised to 22 °C.

In this study, we performed a pathogenicity study, including a larger panel of betanodaviruses and taking into consideration the genetic variability of the viruses, the host of origin of these isolates (European sea bass, sea bream, largemouth black bass and Senegalese sole) and the host environment (marine or fresh water).

Results supported by robust statistical analysis confirmed the pathogenicity, to different degrees, of all the selected betanodavirus isolates. This highlighted the potential occurrence of outbreaks in

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Table 3 Real-time RT-PCR and virus isolation results obtained on samples collected at 8, 44 and 68 days post-infection

ID of the isolate	Virus isolation results 8 d.p.i.	Real-time RT-PCR results (CP of positive samples) 8 d.p.i.	Virus isolation results 44 d.p.i.	Real-time RT-PCR results (CP of positive samples) 44 d.p.i.	Virus isolation results 68 d.p.i.	Real-time RT-PCR results (CP of positive samples) 68 d.p.i.
378/I02 lab. reference isolate	+	10.23	+	15.16	+	17.61
389/I96	+	12.83	+ (II subcol.)	24.16	+ (II subcol.)	33.32
367-2/I05	+	17.26	+	19.28	+	25.93
367-3/I05	+	9.77	+	14.91	+	23.66
324/I06	+	11.91	+ (II subcol.)	19.67	+	26.51
82/I07	+ (II subcol.)	27.37	+ (II subcol.)	22.94	+ (II subcol.)	21.48
243/I09	+	10.00	+	18.64	+ (II subcol.)	23.49
250/I09	+	21.50	+ (II subcol.)	16.88	+ (II subcol.)	26.57
283/I09	+	11.18	+	17.01	+	26.88
484-2/I09	+ (II subcol.)	35.02	+ (II subcol.)	25.26	–	neg.
Negative control	–	neg.	–	neg.	–	neg.

+, cpe detected; –, no cpe detected; neg., negative; II subcol., cpe detected during subcultivation.

European sea bass caused by VER/VNN strains originating from different species and characterized by distinct genetic profiles.

The final cumulative mortality observed in the experimental groups ranged between 7.5% (infection with 82/I07-RGNNV/SJNNV, sea bream) and 36.25% (infection with 283/I09-RGNNV, sea bass). In the majority of cases, the mortality caused by a given strain was proportionate with the increase in T°C. This results could be correlated with results obtained *in vitro* showing that the RGNNV genotype has a higher optimal growth temperature (25–28 °C) compared with SJNNV (Iwamoto *et al.* 2000; Hata *et al.* 2010). Reverse genetics nodavirus reassortant strains have been shown to have an optimal temperature range for *in vitro* growth between 20 and 25 °C (Hata *et al.* 2010). This is in agreement with our results, where three of four of the reassortant-tested strains (82/I07-RGNNV/SJNNV; 367-2/I05-RGNNV/SJNNV and 389/I96-SJNNV/RGNNV) had a mortality rate with the increase in water temperature. Nevertheless, this study has revealed the existence of notable differences and exceptions among the same VER/VNN genotype in response to changes in T°C, thus underlining that the course of the disease in European sea bass can be influenced either by the effect of adverse environmental conditions (i.e. high T°C) or the intrinsic characteristics of the strains. Thus, strains exhibited significant differences in the cumulative mortality under the same experimental conditions, indicating an association between the pathogenicity and the viral phenotype and/or genotype. In some instances, this association appeared not related to T°C, as in the case of strains 367-3/I05-RGNNV and 484-2/I09-SJNNV. Furthermore, while some strains were capable of causing high mortality at 20 °C (e.g. strains 283/I09-RGNNV and 250/I09-RGNNV/SJNNV), other ones (e.g. strains 82/I07 and 367-2/I05-RGNNV/SJNNV, both) caused increased mortality only at the highest water temperature of 25 °C and after the acute clinical phase. Nevertheless, in these cases, the virus was detected in the target organ of the fish suggesting possible long-term pathogenic effects.

Our results seem to indicate that no robust relationship can be established between nodavirus genotype and its pathogenicity in sea bass, the main target species in Mediterranean aquaculture. Whether this hypothesis is related to the replication capacity of different viral genotypes at different temperatures, as demonstrated *in vitro* (Hata *et al.* 2010), or to pathogenicity-associated markers not investigated with the sequencing procedure applied in this work is still a matter which needs further investigations. The second hypothesis seems to be supported by sequence analysis results of viral strains used by Breuil *et al.* (2001). Even though in this study no genetic characterization was performed and the strains isolated from European sea

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bass were distinguished on the basis of the geographical site of isolation, further investigations on the RNA 2 sequence of those strains (Thiéry, Arnauld & Delsert 1999; Panzarin *et al.* 2012) attributed both of them to the RGNNV genotype.

Isolate 243/I09-RGNNV was originally obtained from diseased largemouth black bass reared in a freshwater environment (Bovo *et al.* 2011), suggesting that salinity does not represent an essential factor for the development of the disease and supporting the hypothesis that pathogenic betanodaviruses may circulate freely between the freshwater and marine environments.

Strains 324/I06-RGNNV, 250/I09-RGNNV/SJNNV and 82/I07-RGNNV/SJNNV isolated from gilthead sea bream managed to induce the disease in experimentally infected European sea bass, confirming the possibility that this host, even if asymptomatic, may act as a vector and transmit the infection to susceptible species as previously suggested (Castric *et al.* 2001).

The strain 484-2/I09-SJNNV was originally isolated from asymptomatic Senegalese sole during routine screening activities (Prof. F. Padros, pers. comm). In this study, we have demonstrated for the first time how a representative of this genotype can infect European sea bass and induce mortality in this species, thus indicating a potential epidemiological role for Senegalese sole in harbouring, although asymptomatically, pathogenic viruses for European sea bass. Nevertheless, the capacity of this strain to replicate in this host and in these experimental conditions seems to be lower than that of the other betanodavirus strains used in this study, as the isolation of the virus from fish samples collected at selected time points was achieved only in the subculture, both at 8 and 44 days p.i. Finally, at 68 days p.i., viral growth was no longer evident, and the CP value was over the cut-off point. Thus, considering that in all the remaining groups, the virus was detected for at least 68 days, it is possible to speculate that the biological cycle of this SJNNV strain in European sea bass could be shorter. These results are consistent with other reports that describe low host specificity (Thiéry *et al.* 2004) of betanodavirus isolates detected within European marine fish. Indeed, testing more SJNNV strains in sea bass would be of extreme interest and the aim of a future work.

It is noteworthy that real-time RT-PCR performed on untargeted collected healthy fish from the majority of infected groups (with the exception of 82/I07-RGNNV/SJNNV; 484-2/I09-SJNNV) at 8 d.p.i. indirectly indicated high viral loads (i.e. low CP values – Table 3) and the presence of infective viral particles – as confirmed by virus isolation – before the appearance of clinical signs at 9–10 days after the infection, depending on the strain.

Viral isolation as well as viral titration performed on dead/moribund fish would have provided more information on the samples than those provide by real-time RT-PCR results only. Due to the small size of the fish used for this trial (0.2 g), it was not possible to perform virus isolation and PCR from each single sample. As we confirmed the presence of viral RNA in the target organ of each dead fish collected, with the exception of the unique fish dead on 64 day p.i. in the 484-2/I09-SJNNV infected group, and with most fish showing typical VER/VNN clinical signs, we can speculate that all the viruses used for this trial developed in an active infection in fish, even if we did not reisolate them from each dead fish.

A final observation should concern the weight gain. Data obtained in this experiment indicate, in agreement with field observations (Dr. C. Zarza, pers. comm), that a disease outbreak is characterized not only by increasing mortality but also by a marked reduction in fish growth and increasing differences in weight/size of the affected fish. This represents an indirect but significant cost loss which is often underestimated. This observation is true for all the betanodavirus strains tested in our work and is supported by strong statistical evidence.

Our results demonstrate that all betanodavirus strains tested are pathogenic for European sea bass juveniles (0.2 g), even though they have a reservoir in fish species apparently more resistant to the disease. No strong correlation between genotype, water temperature, host of origin and pathogenicity has been observed in this work, suggesting the existence of determinants of pathogenicity other than those investigated.

Interestingly, the increase in the mortality rate was not related to the increase in water temperature for all strains. Notably, even in the case of a low mortality rate, the indirect damage (i.e. reduction in growth performance, increase in differences in size beween the survivors and increase in number of poor-quality fish) caused by the infection is severe.

These results pose the basis for further investigations which are needed to fully understand *in vivo*

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pathogenicity mechanisms and to assess the impact of the disease in aquaculture production.

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Chapter 9

Discussion and conclusions

Aquaculture production in the Mediterranean region has dramatically grown since its inception, with an increase in production from 487.488 tons in 1995 to 1.228.457 tons in 2007. In particular, the farming of marine fish species has undergone a rapid expansion, with Egypt, France, Greece, Italy, Spain and Turkey and as the main top producers. In the last two decades, the boost of Mediterranean mariculture has mainly been due to the intense farming high value species such as turbot (*Scophthalmus maximus*), gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) (both adult and juveniles) and sole (*Solea senegalensis* and *Solea solea*) (FAO 2010; FEAP 2015). However, rearing aquaculture species at high densities under artificial conditions gives considerable risks of losses from outbreaks of infectious diseases. Viral nervous necrosis (VNN) is considered as one of the most devastating viral threats for marine fish farming and, undoubtedly, represents a major problem for the Mediterranean mariculture. In agreement with previous studies (Olveira et al., 2008; Toffolo et al., 2007), an epidemiological survey carried out in our laboratory highlighted that different betanodavirus genotypes are currently circulating in this region (Panzarin et al., 2012). The RGNNV is the most spread genotype in the Mediterranean basin, extensively circulating in different countries, both in wild and farmed fish populations, while the SJNNV was detected only in Spain in farmed *Solea senegalensis.* Besides, a number of viral strains harbouring the RNA1 gene of the RGNNV and the SJNNV-RNA2 (i.e. reassortant RGNNV/SJNNV) were identified in farmed fish. Our phylogenetic study showed that a major geographic subdivision has been observed among betanodaviruses, as well as a strong clustering depending on the farming status of the host of origin (wild or reared). However, the existence of epidemiological connections among different fish populations has been highlighted. In particular, the occurrence of viral exchanges between feral and reared animals has also been reported in two epidemiological investigations carried out in wild groupers in southern Italy and northern Tunisia (**Chapters 2 and 3**). Although it is almost impossible to precisely assess the extent of a disease outbreak in feral fish, it was clear that VNN involved a high number of animals, and the high water temperature seemed to have played a key role in determining the severity of the phenomenon. Groupers mortality due to betanodavirus infection is certainly an ecological problem of major concern, as some *Epinephelus* species are considered endangered. However, it is also noteworthy that a number of specimens were collected in close proximity of intensively

farmed areas. Interestingly, we have observed a high (if not identical) sequence similarity between viral strains isolated from wild groupers and farmed sea bass sampled in the same or in previous years. Such an observation is highly suggestive of a persistent circulation of betanodavirus in the Mediterranean region and of the existence of viral exchanges between feral and reared fish. Unfortunately, our data do not shed light on the transmission route of the infection, nor do they clarify whether the virus spreads from the wild environment into the farms or viceversa, which is still a debated matter. In this framework, the development of easily accessible tools for genetic and epidemiological data sharing is of great importance, not only to track and trace betanodavirus spread, but also to report the emergence of new viral strains with potentially diverse ecological and phenotypic features. For this purpose and in collaboration with the European Union Reference Laboratory for Fish Diseases (EURL-FISH), we have developed a free online database (**Chapter 4**) available at http://www.fishpathogens.eu/noda. The database collates sequence, epidemiological and biological information related to new disease outbreaks and is implemented with a BLAST-based search function and phylogenetic reconstruction tool, which enable the user to compare a query sequence with all the inventoried sequences uploaded in the database. Such analyses may suggest whether the phenotype of a given genetic strain is altered in comparison to other previously established genotypes, or whether a virus from an ongoing epidemic is a persistently circulating strain or if it represents a new introduction.

Although epidemiological investigations are of crucial importance to plan adequate control strategies for VNN, early diagnosis remains one of the most effective measures to prevent viral spread. Biomolecular techniques such as real-time PCR, couple high performances in terms of sensitivity and specificity with shorter processing and turn-around time. The majority of the assays developed so far were designed to target the RNA2 genetic segment (Dalla Valle et al., 2005; Hick et al., 2010; Hodneland et al., 2011; Panzarin et al., 2010). Notably, fish which have survived the infection, can act as healthy carriers and the diagnosis of betanodavirus in chronically infected fish might be challenging due to the low viral load and, consequently, to sensitivity limits of the method. For this reason, the development of an independent assay targeting the RNA1 gene, to be used as a confirmatory test for those samples yielding doubtful results, is needed. In **Chapter 5** we have described a one-step TaqMan-based real-time PCR protocol designed to detect the

four known betanodavirus genotypes as well as the newly emerged reassortants. Validation data showed that the assay is rapid, specific, sensitive and robust, and has a potential for being applied as a routine method to test a variety of host species originating from different geographic areas.

While biomolecular techniques are well established and widely used for the detection of betanodavirus, serological assays are not recognized as suitable methods for routine diagnosis of VNN, despite their great potential for the screening of broodstock. The limited use of serological methods is mainly due to the scarce knowledge of the humoral response of fish to betanodavirus infection and, most of all, of the antigenic properties of fish nodaviruses. As a matter of fact, nowadays very little is known about the immunoreactivity of different betanodavirus genotypes, and a more comprehensive knowledge on the serological properties of these viruses is needed to implement adequate control strategies against VNN. In **Chapter 6**, by means of serum neutralization tests (SN) we have successfully serotyped unknown field samples and provided a complete description of the serological relationships among different betanodavirus genotypes, including the cold-water strains and the reassortants, for which no information was available. Differently from what previously reported by Mori et al. (2004), we observed that the BFNNV genotype do not cluster within serotype C, but forms a unique serogroup together with the TPNNV genotype and the cold-water strains from Atlantic cod and Atlantic halibut (serotype B). Our work confirms the diverse antigenic reactivity of the RGNNV and the SJNNV genotypes (constituting serotypes C and A, respectively). Interestingly, data obtained demonstrate that the serogrouping of the reassortant strains RGNNV/SJNNV and SJNNV/RGNNV depends on the donor genotype of their RNA2 segment, which indicates that the coat protein gene is the major genetic determinant of betanodavirus serological profiles. To better understand the molecular basis of betanodavirus immunoreactivity, we focused our attention on RGNNV and SJNNV, the genetic types currently circulating in the Mediterranean. Notably, due to their different serological reactivity, these genotypes are a suitable model for the identification of the molecular traits responsible for their diverse phenotypes. Immunostaining of *in vitro* expressed wild type and chimeric capsid proteins between the RGNNV and the SJNNV genotypes suggests that the C-terminal part of the capsid protein is involved in the antigenic diversity of these betanodavirus species. The amino acid residues determining RGNNV and SJNNV antigenic diversity were

mapped at positions 217-256 and 257-341, respectively. Interestingly, SN of chimeric viruses obtained through the reverse genetics technology indicated that the C-terminal portion of the capsid protein contain the neutralizing epitopes. Data reported in **Chapter 6** provides an exhaustive overview of the serological relationship existing among different viral genotypes and paves the way for the implementation of target serological diagnosis as an alternative strategy for viral typing, especially for those laboratories which are not equipped for advanced genetic analysis. Notably, the low cross-reaction among different betanodavirus serotypes (namely A and C) observed in the present study must be kept into account when serological diagnosis is applied, particularly in those areas where more than one betanodavirus genotype is circulating (i.e. the Mediterranean basin) (Panzarin et al., 2012). Furthermore, the genotype-specific reactions, suggest that a multivalent vaccine is needed to prevent infection caused by any betanodavirus genotype. Indeed, although early diagnosis and prevention through the adoption of proper biosafety measures are certainly appropriate to prevent the spread of the infection, vaccination remains the elective control-method for a sound long-term sustainable economic-, environmental- and ethical basis, and field experts agree that an efficient VNN vaccine would be needed. Therefore, the study of immunoreactivity of the virus causing VNN is fundamental for the rational design of cross-protective commercial vaccines with positive side effects on marine fish farming of susceptible species. In this framework, our data have shed some light onto the molecular determinants responsible for betanodavirus immunoreactivity and may be used for future development of cross-protective vaccines against VNNV.
Another key aspect of viral phenotype which has been object of investigation in the present thesis is betanodavirus temperature sensitivity. In **Chapter 7**, we have studied the viral fitness *in vitro* of the RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV under different incubation temperatures by developing growth curves. Obtained data demonstrated that the severity of the cytopathic effect and viral replication are dependent on both the genetic type and the incubation temperature. Interestingly, while at lower temperatures (15, 20 and 25°C) the extent of CPE and the viral replication were slightly comparable among diverse betanodavirus strains, at 30°C phenotypic differences appeared to be macroscopic. Indeed, at this incubation temperature the RGNNV and the RGNNV/SJNNV viruses showed a more severe cytopathic effect on cell monolayers, as well as a sharp increase of viral titre indicative of a rapid and efficient replication. On the

contrary, the SJNNV and the SJNNV/RGNNV showed a milder phenotype in terms of CPE and a suboptimal viral growth. In agreement with previous findings by Hata et al. (2010), our data suggest that the RNA1 and/or its encoded protein plays a major role in controlling temperature sensitivity of fish nodaviruses, and that betanodavirus replication is a temperature-sensitive process. It is well known that environmental temperature plays a key role also *in vivo*, determining the disease onset and severity. As a matter of fact, VNN was also known as "summer disease", because of the seasonal occurrence of disease outbreaks both in wild and farmed animals (**Chapters 2 and 3**) (Le Breton et al., 1997). In **Chapter 8**, we have subjected sea bass juveniles to experimental challenge with genetically different betanodaviruses by gradually increasing housing water temperature. The cumulative mortality rate ranged from a minimum of 7.5% to a maximum of 36.25%, which confirmed the susceptibility of sea bass to the infection with all the betanodavirus genotypes currently circulating in Europe. Generally speaking, our data showed that mortality increased with the raise of water temperature and the mortality rate determined by different betanodavirus genotypes seems to reflect their *in vitro* phenotype. An experimental study aimed at better assessing the effect of water temperature (20, 25 and 30°C) on the mortality rate and viral load in the brain of European sea bass experimentally infected with different betanodaviral strains (Toffan et al., manuscript in preparation) shows that the RGNNV species is actually the most pathogenic genotype at high temperatures (30°C) and brains of survivors yielded positive results by real-time PCR at all the housing conditions tested (20, 25 and 30°C). On the other hand, the SJNNV showed a milder phenotype in spite of the housing temperature, and viral genome was detected in brains of fish maintained at 20°C, but not at 25°C and 30°C. Interestingly, the reassortant strains showed low mortality rate under different environmental conditions, but the viral load in the brain was strongly influenced by water temperature and the genetic type of the polymerase gene. Indeed, the RNA of the SJNNV/RGNNV was detectable in the brains of survivors at 20 and 25°C, but not at 30°C, while viral genome of strain RGNNV/SJNNV was detectable at all the incubation temperatures, as for the RGNNV. Taken together, these data show that nodavirus replication *in vivo* is a composite process dependant on intrinsic genetic features of the viral strains and on extrinsic environmental conditions such as water temperature.

In conclusion, data generated in this thesis allow a better understanding of betanodavirus ecology in the Mediterranean region and provide useful information on some important aspects of viral phenotype, clarifying also the role of genetic reassortment. Besides, our data contribute to the implementation of control strategies to limit the spread of the infection and provide some basic knowledge which might assist the selection of cross-protective vaccines against VNN.

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