Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe

Valentina Panzarina, Alice Fusaro, Isabella Monne, Elisabetta Cappellozza, Pierpaolo Patarnello, Giuseppe Bovo, Ilaria Capua, Edward C. Holmes, Giovanni Cattoli

Abstract

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.

1. Introduction

Viral encephalopathic 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., 1992; Grotmol et al., 1995; Le Breton et al., 1997; 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., 1992; Grotmol et al., 1995; Le Breton et al., 1997; Munday et al., 2002).

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; Cuijil et al., 2006, 2007; Dalla Valle et al., 2001; Skliris et al., 2001; Thiery 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 reassortant betanodavirus, in the form of a RGNNV/SJNNV variant, has been detected in sea bream (Sparus aurata) and Senegalese sole (Solea senegalensis) farmed in Portugal and Spain (Oliveira 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 activities 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 manufacturer’s 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 + Γ4 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, Γ4), 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 (tMRCA) 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 (BMC/MC) 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-
lesser 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 (dN) to synonymous (dS) nucleotide substitutions per site (dN/dS). In all cases dN/dS 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 along 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
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
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.

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.)
The mean rate of nucleotide substitution for the RNA1 gene was $4.89 \times 10^{-4}$ substitution/site/year (95% HPD, 2.92 $\times 10^{-4}$ to 6.97 $\times 10^{-4}$). Notably, a significantly lower rate of nucleotide substitution was found for the RNA2 gene, at $2.42 \times 10^{-4}$ substitution/site/year (95% HPD, 1.07 $\times 10^{-4}$ to 3.99 $\times 10^{-4}$). 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 $d_{s}/d_{a}$ 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 291 fell within the 232–255 amino acid 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 local–specificity (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, Mugiliformes, 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 ($n=32$), 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 Oliveira 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

### Table 1

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

<table>
<thead>
<tr>
<th>RNA1 cluster</th>
<th>RNA2 cluster</th>
<th>Country</th>
<th>Period</th>
<th>N. farmed fish</th>
<th>N. wild fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A</td>
<td>Greece</td>
<td>2005–07</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>–</td>
<td>Italy</td>
<td>2003–09</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>III</td>
<td>–</td>
<td>Italy</td>
<td>2004–05</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>–</td>
<td>Italy</td>
<td>2008–09</td>
<td>7</td>
<td>1*</td>
</tr>
<tr>
<td>IV</td>
<td>E</td>
<td>Croatia</td>
<td>2003–09</td>
<td>9b</td>
<td>18</td>
</tr>
<tr>
<td>V</td>
<td>F</td>
<td>Spain</td>
<td>2005–09</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>–</td>
<td>Spain</td>
<td>2006–07</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>–</td>
<td>Italy</td>
<td>2005</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>VIII</td>
<td>G</td>
<td>Croatia</td>
<td>2001–09</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>XI</td>
<td>–</td>
<td>Spain</td>
<td>2009</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IX</td>
<td>C</td>
<td>Italy</td>
<td>2002–05</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>X</td>
<td>D</td>
<td>Italy</td>
<td>2002–09</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>Italy</td>
<td>2000</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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.
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 dS/dD 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 m², 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 these 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.

References


