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Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs

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1    **Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs**

2    Running title: Phylogenetic analysis of betanodaviruses

3

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26    help.

27    **Abstract**

28    Betanodaviruses are small ssRNA viruses responsible for viral encephalopathy and retinopathy,  
29    otherwise known as viral nervous necrosis, in marine fish worldwide. These viruses can be either  
30    horizontally or vertically transmitted and have been sporadically detected in invertebrates, which  
31    seem to be one of the possible viral sources.

32    Twenty-eight new betanodavirus strains were retrieved in three molluscs species collected from  
33    different European countries between 2008 and 2015. The phylogenetic analyses revealed that  
34    strains retrieved from bivalve molluscs are closely related with viruses detected in finfish in  
35    Southern Europe in the period 2000-2009. Nevertheless, a new betanodavirus strain, markedly  
36    different from the other members of the RGNNV genotype was detected. Such a massive and varied  
37    presence of betanodaviruses in bivalve molluscs greatly stresses the risks of transmission previously  
38    feared for other invertebrates. Bivalve molluscs reared in the same area as farmed and wild finfish  
39    could act as a reservoir of the virus. Furthermore, current European regulations allow relaying  
40    activities and the sale of live bivalve molluscs, which could pose a real risk of spreading  
41    betanodaviruses across different geographic regions. To our knowledge, this is the first study which  
42    focuses on the detection and genetic characterisation of betanodaviruses in bivalve molluscs.

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47    **Keywords:** Betanodavirus, bivalve mollusc, molecular detection, phylogenetic analysis, viral  
48    encephalopathy and retinopathy, nervous necrosis virus.

## 1. Introduction

Betanodaviruses are small ssRNA viruses of the genus *Betanodavirus*, family *Nodaviridae* (Mori et al., 1992; Thiéry et al., 2012) responsible for viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), in several fish species worldwide. The Betanodavirus genome consists of two segments named RNA1 (3.1 KB) and RNA2 (1.4 kb), coding for the RNA-dependent RNA polymerase and the coat protein respectively. Moreover, during virus replication, a subgenomic transcript called RNA3 is originated from the 3' terminus of RNA1 (Mori et al., 1992; Sommerset et al., 2004; Iwamoto et al., 2005; Fenner et al., 2006; Thiéry et al., 2012). Based on the phylogenetic analysis of the T4 variable region within the RNA2 segment, betanodaviruses have been clustered into four genotypes, currently accepted as official species of this genus: *Striped jack nervous necrosis virus* (SJNNV), *Tiger puffer nervous necrosis virus* (TPNNV), *Barfin flounder nervous necrosis virus* (BFNNV) and *Redspotted grouper nervous necrosis virus* (RGNNV) (Nishizawa et al. 1997; Thiéry et al., 2012). An additional genotype clustering outside the four established fish nodavirus species, isolated from *Scophthalmus maximus* and named turbot nodavirus (TNV), has yet to be officially classified (Johansen et al., 2004). Although betanodavirus genotyping is mostly based on RNA2 phylogenetic analysis (Nishizawa et al. 1997), the sequencing of RNA1 has added further information by showing the presence of reassortant strains (Oliveira et al., 2009; Toffolo et al., 2007). As a matter of fact, the presence of reassortant betanodaviruses SJNNV/RGNNV had already been described in sea bass (*Dicentrarchus labrax*) from Italy and Croatia, in the form of a genetic variant containing the RNA1 segment from the SJNNV genotype and the RNA2 molecule from the RGNNV-type (Toffolo et al., 2007). A new reassortant betanodavirus in the form of a RGNNV/SJNNV genetic variant was later detected in sea bream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*) and common sole (*Solea solea*) farmed in Portugal, Spain and Italy (Oliveira et al., 2009; Panzarin et al., 2012).

78 VER is mainly observed in farmed fish, although severe outbreaks were reported in wild fish,  
79 mainly groupers (Gomez et al., 2009; Vendramin et al., 2013). Furthermore, asymptomatic  
80 betanodavirus infection has also been detected in wild fish (Barker et al., 2002; Gomez et al., 2004;  
81 Baeck et al., 2007; Ciulli et al., 2007a; Gomez *et al.* 2008a, Panzarin et al., 2012; Liu et al., 2015).  
82 The occasional presence of betanodaviruses in invertebrates was also detected in the Mediterranean  
83 Sea, South Korea and Japan (Gomez et al., 2006; Gomez et al., 2008b; Gomez et al. 2010; Ciulli et  
84 al. 2010; Panzarin et al., 2012; Fichi et al., 2015). With particular reference to bivalve molluscs,  
85 betanodavirus was also reported in two mussel (*Mytilus galloprovincialis*) samples collected in  
86 Korea and in one sample of clam (*Ruditapes philippinarum*) from Italy (Gomez et al., 2008b;  
87 Panzarin et al., 2012). In truth, the presence of betanodaviruses in Italian clams and French oysters  
88 was reported for the first time in 2010 and preliminary results were presented at the 14th  
89 International Biotechnology Symposium and Exhibition (Ciulli et al., 2010).  
90 As a matter of fact, most of the genetically characterised betanodaviruses detected in invertebrates  
91 belonged to the RGNNV genotype. However, a reassortant RGNNV/SJNNV strain was found in  
92 *Artemia salina* and *Opisthobranchia* (Gomez et al., 2008b; Gomez et al., 2008c; Ciulli et al., 2010;  
93 Panzarin et al., 2012). Overall, a very limited number of studies have been conducted on this topic.  
94 Betanodavirus infection can occur through two pathways: horizontal and vertical transmission. In  
95 addition, interspecies transmission is also possible and genetically related viruses are often detected  
96 in different species. For these reasons, wild fish have been supposedly believed to be a source of the  
97 virus (Gomez et al., 2006; Gomez et al., 2008a; Doan et al., 2017). Similarly, it was assumed that  
98 betanodavirus can be transmitted to finfish through trash fish, which consist of both marine  
99 vertebrates and invertebrates (Gomez et al., 2010). However, several factors can affect the real risk  
100 of betanodavirus transmission from invertebrate to finfish, including the prevalence of the virus in  
101 invertebrate populations and the similarity of viruses detected in different organisms. In this study,  
102 we examined bivalve molluscs reared in different European countries for the presence of  
103 betanodaviruses, in order to assess the prevalence of contaminated specimens and to determine the

104 genetic relatedness of viruses detected in molluscs and in finfish species. Data obtained indicate that  
105 betanodavirus closely related to finfish strains can be detected in bivalve molluscs, which poses a  
106 possible risk for viral spread into new areas/populations.

107

## 108 **2. Materials and Methods**

### 109 **2.1. Bivalve molluscs**

110 The betanodaviruses characterised in this study were obtained from samples collected during a  
111 preliminary survey conducted in 2009, which was intended to investigate the presence of these  
112 viruses in three bivalve mollusc species (Ciulli et al., 2010). A total of 57 batches (19 for each  
113 species) of retail bivalve molluscs were analysed, including a species reared on the seafloor, such as  
114 clam (*Ruditapes philippinarum*) and species usually farmed on the water column, as in the case of  
115 oysters (*Crassostrea gigas*) and mussels (*Mytilus galloprovincialis*). Each species was equally  
116 represented in the sampling batches, which were composed of 30 clams, 10 mussels or 6 oysters.  
117 Bivalve mollusc batches were collected in 2009 directly from the market and originated from  
118 France (oysters), Italy (clams and mussels) and Spain (mussels).

119 Further diagnostic activities on Italian clam (*Ruditapes philippinarum*) were conducted between  
120 September 2012 and May 2015 and consisted in betanodavirus screening of additional 36 batches.

121

### 122 **2.2. RNA extraction, RT-PCR and nested PCR**

123 The mollusc hepatopancreas samples were homogenized and treated with proteinase K (Sigma, St.  
124 Louis, USA); the RNA was then extracted according to the manufacturer's instructions with  
125 NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany). RNA samples were stored at -80 °C  
126 until use.

127 Betanodavirus presence was investigated by a RT-nested PCR method using previously described  
128 primers targeting the viral RNA2 (Ciulli et al., 2007b). Briefly, the first amplification step was  
129 conducted through a one-step RT-PCR assay with primers S6 (5'-ATGGTACGCAAAGGTGATAA

130 GAAA-3') and S7 (5'-GTTTTCCGAGTCAACACGGGT-3') (Ciulli et al., 2006) using the  
131 SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA). The reaction mixture  
132 contained 1x Reaction Mix, 0.8  $\mu$ M of each primer, 0.3  $\mu$ l Superscript III/Platinum Taq enzyme  
133 mix and 3  $\mu$ l RNA in 15  $\mu$ l total volume. The optimal thermal cycling conditions were 45°C for 30  
134 min, 95°C for 2 min, followed by 40 amplification cycles of 94°C for 60 sec, 58°C for 60 sec and  
135 72°C for 60 sec. A final extension was performed at 72°C for 7 min. Nested PCR was conducted  
136 with primers F2 (5'-CGTGTCAGTCATGTGTCGCT-3') and R3 (5'-  
137 CGAGTCAACACGGGTGAAGA-3') (Nishizawa et al., 1994) using the Platinum Taq DNA  
138 polymerase (Invitrogen). The reaction mixture contained 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.25  $\mu$ M  
139 of each primer, 1.25 units of Platinum Taq DNA polymerase, nuclease free water and 1  $\mu$ l of 1:100-  
140 diluted PCR product from the RT-PCR analysis in 25  $\mu$ l total volume. The thermal cycle consisted  
141 of denaturation at 95°C for 5 min and of 40 amplification cycles of 94°C for 30 sec, 56°C for 30 sec  
142 and 72°C for 30 sec. A final extension was performed at 72°C for 7 min. To avoid any cross  
143 contamination, negative controls were run along with all reactions. The results of all RT-PCR and  
144 nested PCR analyses were checked by agarose gel electrophoresis of PCR products along with a  
145 100 bp DNA molecular marker (Invitrogen, Carlsbad, USA).

146 A selection of nine RNA2-positive betanodavirus samples collected between 2012 and 2015 was  
147 also tested for the RNA1 fragment using primers previously described (Toffolo et al., 2007). RT-  
148 PCR was performed with the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad,  
149 USA) using primers VNNV5 (5'-GTTGAGGATTATCGCCAACG-3') and VNNV6 (5'-  
150 ACCGGCGAACAGTATCTGAC-3'). Semi nested PCR was conducted with primers VNNV6 and  
151 VNNV7 (5'-CACTACCGTGTGCTG-3') using the Platinum Taq DNA polymerase (Invitrogen).

152

### 153 2.3. Sequencing and phylogenetic analyses

154 PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim,  
155 Germany) and then sequenced by the Bio-Fab Sequencing Service (Rome, Italy).



RNA1 ( $n = 9$ ) and RNA2 ( $n = 28$ ) nucleotide sequences were aligned and compared with betanodavirus sequences previously obtained from isolates detected in farmed and wild finfish, as well as with betanodavirus reference strains available in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using Clustal W implemented in the BioEdit software (<http://bioedit.software.informer.com/>). More specifically, we used a representative selection of betanodavirus sequences from southern Europe characterised during a previous molecular epidemiology survey (Panzarin et al., 2012). To describe the phylogenetic relationships among the betanodavirus strains detected in bivalve molluscs and finfish, the maximum likelihood (ML) method available in the PhyML program version 3.1 (Guindon et al., 2010) was used. The analysis incorporates the general time-reversible (GTR) model of nucleotide substitution with a gamma distributed rates across sites (four rate categories,  $\Gamma 4$ ) and uses a SPR branch-swapping search procedure (Darriba et al., 2012). One thousand bootstrap replicates were performed to assess the robustness of individual nodes, and only values  $\geq 60\%$  were considered significant. Phylogenetic trees were visualized with the FigTree v1.4 software (<http://tree.bio.ed.ac.uk/software/figtree/>). Amino acid sequences were predicted by the BioEdit software (<http://bioedit.software.informer.com/>). The percentage of pairwise nucleotide and amino acid similarity was calculated with the BioEdit software.

173

### 174 **3. Results**

#### 175 3.1. Virus detection

176 All samples collected during the 2009 survey resulted negative to RT-PCR, while 15 batches out of  
177 57 resulted positive to nested PCR (26.3 %). Only one batch of mussels (*M. galloprovincialis*) out  
178 of nineteen (1/19) was found positive, whereas clams (*R. philippinarum*) and oysters (*C. gigas*)  
179 resulted highly positive to betanodavirus, with 42.1 % (8/19) and 31.6% (6/19) of positive batches,  
180 respectively. All the positive samples except for one (585/2009) were collected between June and  
181 September, 2009.

182 Overall, the 2009 survey allowed to collect six betanodavirus strains from French oysters and eight  
183 from Italian clams; furthermore, one betanodavirus was detected in a mussel batch, but no data on  
184 the geographic origin of this sample were available (Table 1). Between 2012 and 2015 the  
185 diagnostic activity on Italian clams allowed to collect additional 12 betanodavirus strains (Table 1).  
186 A strain from mussels collected in Sicily in 2008 at the Istituto Zooprofilattico Sperimentale della  
187 Sicilia (Italy) was also included in the phylogenetic analysis (Table 1).

188

### 189 3.2. Sequencing and phylogenetic analysis

190 The ML phylogenetic trees inferred for the RNA1 and RNA2 genes of the viruses collected from  
191 bivalve molluscs between 2008 and 2015 revealed that all the betanodaviruses detected in this study  
192 were RGNNV (Figs. 1, 2), with the exception of strain 681M/2009 for which a high genetic and  
193 amino acid diversity was observed (see below) (Fig. 2 and Table 2).

194 The RNA2 analysis highlighted that betanodaviruses from bivalve molluscs clustered in 2 different  
195 subgroups (B, E), identified in a previous work (Panzarin et al., 2012) and that included finfish  
196 viruses with high variability in terms of year of detection, host species and fish status (wild/farmed).  
197 Subgroup E included betanodaviruses detected in the period 2009-2014 from Italian clams and in  
198 one French oyster sample. Interestingly, subgroup E included also a viral strain previously detected  
199 in *R. philippinarum* (285.13.2009, GenBank accession number JN189993). On the other hand,  
200 subgroup B included only viral strains detected in Italian clams (2009-2014). An additional  
201 subgroup, herein arbitrarily named H, was identified in the RNA2 phylogenetic tree. This cluster  
202 was well supported by bootstrap analysis and included viruses detected in four French oysters and  
203 in one Italian mussel (Sicily). The remaining six betanodavirus strains detected in Italian clams in  
204 2014 and 2015, together with strain 681M/2009 from a mussel sample, do not belong to any  
205 previously described subgroup (Panzarin et al., 2012). Notably, the RNA2 sequence of this latter  
206 virus is significantly different from the one of the RGNNV genogroup, which may suggest the  
207 existence of a new candidate betanodavirus species.

208 The analysis of the RNA1 showed that betanodaviruses detected in Italian clams clustered in 3  
209 distinct subgroups, according to the genetic subdivision suggested by Panzarin et al. (2012), namely  
210 II, IV and X. By comparing betanodaviruses from Italian clams with viruses previously included in  
211 these groups, we noticed that they actually clustered with viruses isolated from finfish during the  
212 period 1996-2009 in different countries of the Mediterranean basin, regardless of the host species  
213 and the fish status (wild/farmed).

214 Failure of sample preservation prevented us from performing the RNA1 sequencing for strain  
215 681M/2009 and for all the other viruses detected before 2012.

216 The betanodaviruses detected in bivalve molluscs showed nucleotide and amino acid identities  
217 higher than 88.9% and 86.1%, respectively, with the exception of strain 681M/2009, which showed  
218 a nucleotide and amino acid identity lower than 74.7% and 82.9%, respectively. The percentage of  
219 pairwise nucleotide and amino acid similarity with the RNA2 of the four betanodavirus genotypes  
220 are reported in Table 2. Apart from strain 681M/2009, all the viruses detected in bivalve molluscs  
221 showed high nucleotide and amino acid identities with the RGNNV genotype, ranging between 89.6  
222 and 99.6% and between 87.2 % and 100.0% respectively. On the contrary, the nucleotide and amino  
223 acid identities with other genotypes such as BFNNV, SJNNV and TPNNV were lower than 76.8,  
224 65.8 and 65.5% and than 85.1, 68.7 and 70.8%, respectively. Strain 681M/2009 showed nucleotide  
225 and amino acid identities of 75.0% and 80.8% with RGNNV and of 72.2% and 79.7% with  
226 BFNNV. These values were lower than those between genotypes RGNNV and BFNNV (76.1%  
227 nucleotide identity and 85.1% amino acid identity).

228

#### 229 **4. Discussion**

230

231 To our knowledge, this is the first study focusing on betanodavirus in bivalve molluscs. Twenty-  
232 eight new viral strains which had been collected in different years, from three bivalve molluscs  
233 species, in several European countries. were genetically characterised.

234 The sporadic presence of betanodaviruses in marine invertebrate had already been reported (Gomez  
235 et al., 2008b; Ciulli et al., 2010; Gomez et al., 2010; Panzarin et al., 2012).

236 In our study, betanodaviruses were found in samples collected over a long period of time (2008-  
237 2015), in different European countries and belonging to three mollusc species, showing a consistent  
238 presence of this virus in the invertebrate hosts. In particular, clams collected in north-eastern Italy  
239 over a 7-year period turned out to be positive for betanodavirus. Moreover, a different prevalence  
240 was shown among bivalve mollusc species; clams seemed to be more frequently contaminated than  
241 oysters and mussels. The presence of the virus in bivalve molluscs might be a natural consequence  
242 of their biology. Bivalve molluscs are obligate filter feeders and can accumulate particles, including  
243 viruses, from the surrounding water (Serratore et al., 2014). The fossorial behaviour of clams could  
244 favour the virus-host contact and viral retention, compared to suspended farming methods used for  
245 oysters and mussels. However, the geographical origin may also have influenced the different  
246 prevalence observed in the bivalve mollusc species object of our study.

247 Phylogenetic analysis of both RNA1 and RNA2 fragments of betanodaviruses from bivalve  
248 molluscs showed a wide range of strains, mainly belonging to the RGNNV genotype, which is the  
249 most frequently reported in Europe.

250 However, the RNA2 genetic analysis showed the presence of one atypical betanodavirus  
251 (681M/2009) retrieved from a mussel batch, showing the highest nucleotide and amino acid  
252 similarity with the RGNNV even if markedly different from the other members of this genotype.

253 For this reason, strain 681M/2009 might represent a new betanodavirus species or a new subgroup  
254 of the RGNNV genotype.

255 The phylogenetic analysis of the viruses detected in bivalve molluscs showed no correlation with  
256 their host species and geographical origin, as they clustered with viruses detected in Italian clams  
257 and mussels, as well as in French oysters.

258 The comparison between the viruses from bivalve molluscs and the betanodaviruses isolated from  
259 finfish in Southern Europe (Panzarin et al., 2012) showed the circulation of genetically similar

260 viruses in finfish and in bivalve molluscs. Similarly, to what reported in a previous study, several  
261 subgroups were identified in RNA1 and RNA2 phylogenetic trees within the RGNNV genotype.  
262 Most of these clusters included both bivalve molluscs and finfish viruses, with different geographic  
263 origin, year of isolation and host status (wild/farmed). The bivalve mollusc betanodaviruses did not  
264 cluster separately from finfish viruses, but rather they reflected the epidemiological patterns of  
265 betanodavirus circulating in finfish in Southern Europe.  
266 Furthermore, some recent bivalve mollusc viruses clustered with finfish viruses which had been  
267 detected several years before (1996-2000), thus demonstrating the persistent circulation of these  
268 viruses.  
269 It has recently been demonstrated that clams can accumulate viable RGNNV and release it via  
270 faecal matter and filtered water into the surrounding environment, putting susceptible cohabiting  
271 cultured fish at risk (Volpe et al., 2017). For this reason, it is of utmost importance to investigate the  
272 features of viruses naturally associated with bivalve molluscs. A previous study revealed the  
273 presence of a betanodavirus closely related to RGNNV in marine invertebrate (Japanese common  
274 squid *Todarodes pacificus*), which showed a high pathogenicity to finfish and caused severe  
275 mortalities after intra-muscular challenge (Gomez et al., 2010).  
276 The present study shows that betanodaviruses detected in bivalve molluscs are genetically similar to  
277 those of finfish. The release of these viruses from bivalve molluscs may represent a dangerous  
278 source of viruses for finfish.

279

## 280 **5. Conclusions**

281 The outcome of our study showed the wide diffusion of betanodaviruses in bivalve molluscs, maybe  
282 even greater than expected. Phylogenetic analyses showed that strains detected both in bivalve  
283 molluscs and finfish were closely related and that betanodaviruses retrieved from bivalve molluscs  
284 in different European countries between 2008 and 2015 mimicked the epidemiological patterns of

285 betanodaviruses collected from finfish in Southern Europe between October 2000 and November  
286 2009.

287 Moreover, the nucleotide and amino acid sequence analysis of strain 681M/2009 showed the  
288 existence of a new betanodavirus strain, which could possibly represent either a novel  
289 betanodavirus species or a new RGNNV subgroup.

290 Such a massive and varied presence of betanodaviruses in bivalve molluscs greatly stresses the risks  
291 of transmission previously feared for other invertebrates. Consequently, the accumulation and  
292 release of viable virus by bivalve molluscs, acting as virus carriers, should lead to us paying more  
293 attention to the genetic characterization of the viruses naturally associated with these invertebrates.

294 As a matter of fact, current European regulations allow the sale of live bivalve molluscs and  
295 relaying activities, which could pose a real risk of spreading betanodaviruses across different  
296 geographic regions.

297

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**Table 1.** Details of betanodavirus strains retrieved from bivalve molluscs and used for phylogenetic analysis.

Sample names	Species	Years of sampling	Origin	Genbank accession numbers	
				RNA 1	RNA 2
PA3M	Mussel	2008	Italy, Sicily	nd	MG195159
681M	Mussel	2009	Not available	nd	MG195160
585O	Oyster	2009	France, Atlantic Ocean	nd	MG195161
651O,	Oyster	2009	France, Atlantic Ocean	nd	MG195162
664O	Oyster	2009	France, Atlantic Ocean	nd	MG195163
666O	Oyster	2009	France, Atlantic Ocean	nd	MG195164
672O	Oyster	2009	France, Atlantic Ocean	nd	MG195165
686O	Oyster	2009	France, Atlantic Ocean	nd	MG195166
628C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195167
629C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195168
651C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195169
667C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195170
671C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195171
676C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195172
680C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195173
684C	Clam	2009	Italy, Northern Adriatic Sea	nd	MG195174
919C	Clam	2012	Italy, Northern Adriatic Sea	MG195187	MG195175
76C	Clam	2014	Italy, Northern Adriatic Sea	nd	MG195175
79C	Clam	2014	Italy, Northern Adriatic Sea	MG195188	MG195177
133C	Clam	2014	Italy, Northern Adriatic Sea	MG195189	MG195178
134C	Clam	2014	Italy, Northern Adriatic Sea	MG195190	MG195179
135C	Clam	2014	Italy, Northern Adriatic Sea	MG195191	MG195180
229C	Clam	2014	Italy, Northern Adriatic Sea	nd	MG195181
271C	Clam	2014	Italy, Northern Adriatic Sea	MG195192	MG195182
272C	Clam	2014	Italy, Northern Adriatic Sea	nd	MG195183
38C	Clam	2015	Italy, Northern Adriatic Sea	MG195193	MG195184
39C	Clam	2015	Italy, Northern Adriatic Sea	MG195194	MG195185
58C	Clam	2015	Italy, Northern Adriatic Sea	MG195195	MG195186

nd: not determinated

**Table 2.** Comparisons of nucleotide and amino acid sequences of a RNA2 fragment, which include the variable region of betanodaviruses detected in bivalve molluscs with reference to betanodavirus strains (RGNNV: AY324870; BFNNV: EU826138; SJNNV: AB056572; TPNNV: EU236149; Thiery et al., 2012). Percentage of pairwise nucleotide and amino acid similarity are shown.

		RGNNV		BFNNV		SJNNV		TPNNV	
		nt	aa	nt	aa	nt	aa	nt	aa
	<b>38C/2015</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>39C/2015</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>58C/2015</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>76C/2014</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>79C/2014</b>	91.1	89.3	71.8	82.9	64.4	68.7	64.8	70.8
	<b>133C/2014</b>	98.5	100	76.8	85.1	65.5	67.7	64.8	69.7
	<b>134C/2014</b>	98.9	98.9	76.1	84.0	65.1	66.6	64.4	68.7
	<b>135C/2014</b>	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
	<b>229C/2014</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>271C/2014</b>	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
	<b>272C/2014</b>	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
	<b>919C/2012</b>	98.5	100	76.8	85.1	64.8	67.7	64.8	69.7
	<b>585O/2009</b>	93.2	92.5	72.9	81.9	64.4	64.5	60.6	66.6
	<b>628C/2009</b>	89.6	87.2	71.1	79.7	64.8	68.7	63.7	69.7
	<b>629C/2009</b>	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
	<b>651O/2009</b>	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
	<b>651C/2009</b>	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
	<b>664O/2009</b>	92.8	91.4	72.5	80.8	64.1	63.5	60.6	65.6
	<b>666O/2009</b>	92.5	91.4	72.2	81.9	64.4	64.5	60.6	65.6
	<b>667C/2009</b>	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
	<b>671C/2009</b>	98.5	100	76.1	85.1	65.5	67.7	64.8	69.7
	<b>672O/2009</b>	98.5	100	76.8	85.1	64.8	67.7	65.5	69.7
	<b>676C/2009</b>	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
	<b>680C/2009</b>	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
	<b>681M/2009</b>	75.0	80.8	72.2	79.7	65.8	68.7	65.1	68.7
	<b>684C/2009</b>	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
	<b>686O/2009</b>	93.2	92.5	72.9	81.9	64.4	64.5	60.6	66.6
	<b>PA3M/2008</b>	93.5	94.6	73.6	81.9	63.7	65.6	62.0	67.7

460 Figure legends

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462 Figure 1. ML phylogenetic tree based on partial RNA1 nucleotide sequences (419 bp). Strains  
463 characterised in this study are shown in bold. Sequences retrieved from GenBank are reported with  
464 the isolate name and accession number. The genotype subdivision according to Nishizawa et al.  
465 (1997) is shown at the main branches. RNA1 genetic clusters (I-XI) are highlighted. Bootstrap values  
466 > 60% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per  
467 site. The scale bar is reported.

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469 Figure 2. ML phylogenetic tree based on partial RNA2 nucleotide sequences (281 bp). Strains  
470 characterised in this study are shown in bold. Sequences retrieved from GenBank are reported with  
471 the isolate name and accession number. The genotype subdivision according to Nishizawa et al.  
472 (1997) is shown at the main branches. RNA2 genetic clusters (A-H) are highlighted. Bootstrap values  
473 > 60% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per  
474 site. The scale bar is reported.



