Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs

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Running title: Phylogenetic analysis of betanodaviruses

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Abstract

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

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

Keywords: Betanodavirus, bivalve mollusc, molecular detection, phylogenetic analysis, viral 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 (Olveira 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 (Olveira et al., 2009; Panzarin et al., 2012).

VER is mainly observed in farmed fish, although severe outbreaks were reported in wild fish, mainly groupers (Gomez et al., 2009; Vendramin et al., 2013). Furthermore, asymptomatic betanodavirus infection has also been detected in wild fish (Barker et al., 2002; Gomez et al., 2004; Baeck et al., 2007; Ciulli et al., 2007a; Gomez *et al.* 2008a, Panzarin et al., 2012; Liu et al., 2015). The occasional presence of betanodaviruses in invertebrates was also detected in the Mediterranean Sea, South Korea and Japan (Gomez et al., 2006; Gomez et al., 2008b; Gomez et al. 2010; Ciulli et al. 2010; Panzarin et al., 2012; Fichi et al., 2015). With particular reference to bivalve molluscs, betanodavirus was also reported in two mussel (*Mytilus galloprovincialis*) samples collected in Korea and in one sample of clam (*Ruditapes philippinarum*) from Italy (Gomez et al., 2008b; Panzarin et al., 2012). In truth, the presence of betanodaviruses in Italian clams and French oysters was reported for the first time in 2010 and preliminary results were presented at the 14th International Biotechnology Symposium and Exhibition (Ciulli et al., 2010).

As a matter of fact, most of the genetically characterised betanodaviruses detected in invertebrates belonged to the RGNNV genotype. However, a reassortant RGNNV/SJNNV strain was found in *Artemia salina* and *Opistobranchia* (Gomez et al., 2008b; Gomez et al., 2008c; Ciulli et al., 2010; Panzarin et al., 2012). Overall, a very limited number of studies have been conducted on this topic.

Betanodavirus infection can occur through two pathways: horizontal and vertical transmission. In addition, interspecies transmission is also possible and genetically related viruses are often detected in different species. For these reasons, wild fish have been supposedly believed to be a source of the virus (Gomez et al., 2006; Gomez et al., 2008a; Doan et al., 2017). Similarly, it was assumed that betanodavirus can be transmitted to finfish through trash fish, which consist of both marine vertebrates and invertebrates (Gomez et al., 2010). However, several factors can affect the real risk of betanodavirus transmission from invertebrate to finfish, including the prevalence of the virus in invertebrate populations and the similarity of viruses detected in different organisms. In this study, we examined bivalve molluscs reared in different European countries for the presence of betanodaviruses, in order to assess the prevalence of contaminated specimens and to determine the

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

2. Materials and Methods

2.1. Bivalve molluscs

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

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

2.2. RNA extraction, RT-PCR and nested PCR

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

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

GAAA-3') and S7 (5'-GTTTTCCGAGTCAACACGGGT-3') (Ciulli et al., 2006) using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA). The reaction mixture contained 1x Reaction Mix, 0.8 µM of each primer, 0.3 µl Superscript III/Platinum Taq enzyme mix and 3 µl RNA in 15 µl total volume. The optimal thermal cycling conditions were 45°C for 30 min, 95°C for 2 min, followed by 40 amplification cycles of 94°C for 60 sec, 58°C for 60 sec and 72°C for 60 sec. A final extension was performed at 72°C for 7 min. Nested PCR was conducted with primers F2 (5'-CGTGTCAGTCATGTGTCGCT-3') and R3 (5'-CGAGTCAACACGGGTGAAGA-3') (Nishizawa et al., 1994) using the Platinum Taq DNA polymerase (Invitrogen). The reaction mixture contained 1X PCR buffer, 1.5 mM MgCl2, 0.25 µM of each primer, 1.25 units of Platinum Taq DNA polymerase, nuclease free water and 1 µl of 1:100-diluted PCR product from the RT-PCR analysis in 25 µl total volume. The thermal cycle consisted of denaturation at 95°C for 5 min and of 40 amplification cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. A final extension was performed at 72°C for 7 min. To avoid any cross contamination, negative controls were run along with all reactions. The results of all RT-PCR and nested PCR analyses were checked by agarose gel electrophoresis of PCR products along with a 100 bp DNA molecular marker (Invitrogen, Carlsbad, USA).

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

2.3. Sequencing and phylogenetic analyses

PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, 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) 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, Γ4) 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 ≥ 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.

3. Results

3.1. Virus detection

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

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

3.2. Sequencing and phylogenetic analysis

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

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

viruses in finfish and in bivalve molluscs. Similarly, to what reported in a previous study, several subgroups were identified in RNA1 and RNA2 phylogenetic trees within the RGNNV genotype. Most of these clusters included both bivalve molluscs and finfish viruses, with different geographic origin, year of isolation and host status (wild/farmed). The bivalve mollusc betanodaviruses did not cluster separately from finfish viruses, but rather they reflected the epidemiological patterns of betanodavirus circulating in finfish in Southern Europe.

Furthermore, some recent bivalve mollusc viruses clustered with finfish viruses which had been detected several years before (1996-2000), thus demonstrating the persistent circulation of these viruses.

It has recently been demonstrated that clams can accumulate viable RGNNV and release it via faecal matter and filtered water into the surrounding environment, putting susceptible cohabiting cultured fish at risk (Volpe et al., 2017). For this reason, it is of utmost importance to investigate the features of viruses naturally associated with bivalve molluscs. A previous study revealed the presence of a betanodavirus closely related to RGNNV in marine invertebrate (Japanese common squid *Todarodes pacificus*), which showed a high pathogenicity to finfish and caused severe mortalities after intra-muscular challenge (Gomez et al., 2010).

The present study shows that betanodaviruses detected in bivalve molluscs are genetically similar to those of finfish. The release of these viruses from bivalve molluscs may represent a dangerous source of viruses for finfish.

5. Conclusions

The outcome of our study showed the wide diffusion of betanodaviruses in bivalve molluscs, maybe even greater than expected. Phylogenetic analyses showed that strains detected both in bivalve molluscs and finfish were closely related and that betanodaviruses retrieved from bivalve molluscs 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	RNA2
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	MG195176
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
38C/2015	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
39C/2015	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
58C/2015	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
76C/2014	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
79C/2014	91.1	89.3	71.8	82.9	64.4	68.7	64.8	70.8
133C/2014	98.5	100	76.8	85.1	65.5	67.7	64.8	69.7
134C/2014	98.9	98.9	76.1	84.0	65.1	66.6	64.4	68.7
135C/2014	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
229C/2014	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
271C/2014	99.6	100	76.5	85.1	65.5	67.7	64.8	69.7
272C/2014	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
919C/2012	98.5	100	76.8	85.1	64.8	67.7	64.8	69.7
585O/2009	93.2	92.5	72.9	81.9	64.4	64.5	60.6	66.6
628C/2009	89.6	87.2	71.1	79.7	64.8	68.7	63.7	69.7
629C/2009	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
651O/2009	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
651C/2009	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
664O/2009	92.8	91.4	72.5	80.8	64.1	63.5	60.6	65.6
666O/2009	92.5	91.4	72.2	81.9	64.4	64.5	60.6	65.6
667C/2009	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
671C/2009	98.5	100	76.1	85.1	65.5	67.7	64.8	69.7
672O/2009	98.5	100	76.8	85.1	64.8	67.7	65.5	69.7
676C/2009	90.7	89.3	71.5	82.9	65.1	68.7	64.8	70.8
680C/2009	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
681M/2009	75.0	80.8	72.2	79.7	65.8	68.7	65.1	68.7
684C/2009	98.9	100	76.5	85.1	65.1	67.7	65.1	69.7
686O/2009	93.2	92.5	72.9	81.9	64.4	64.5	60.6	66.6
PA3M/2008	93.5	94.6	73.6	81.9	63.7	65.6	62.0	67.7

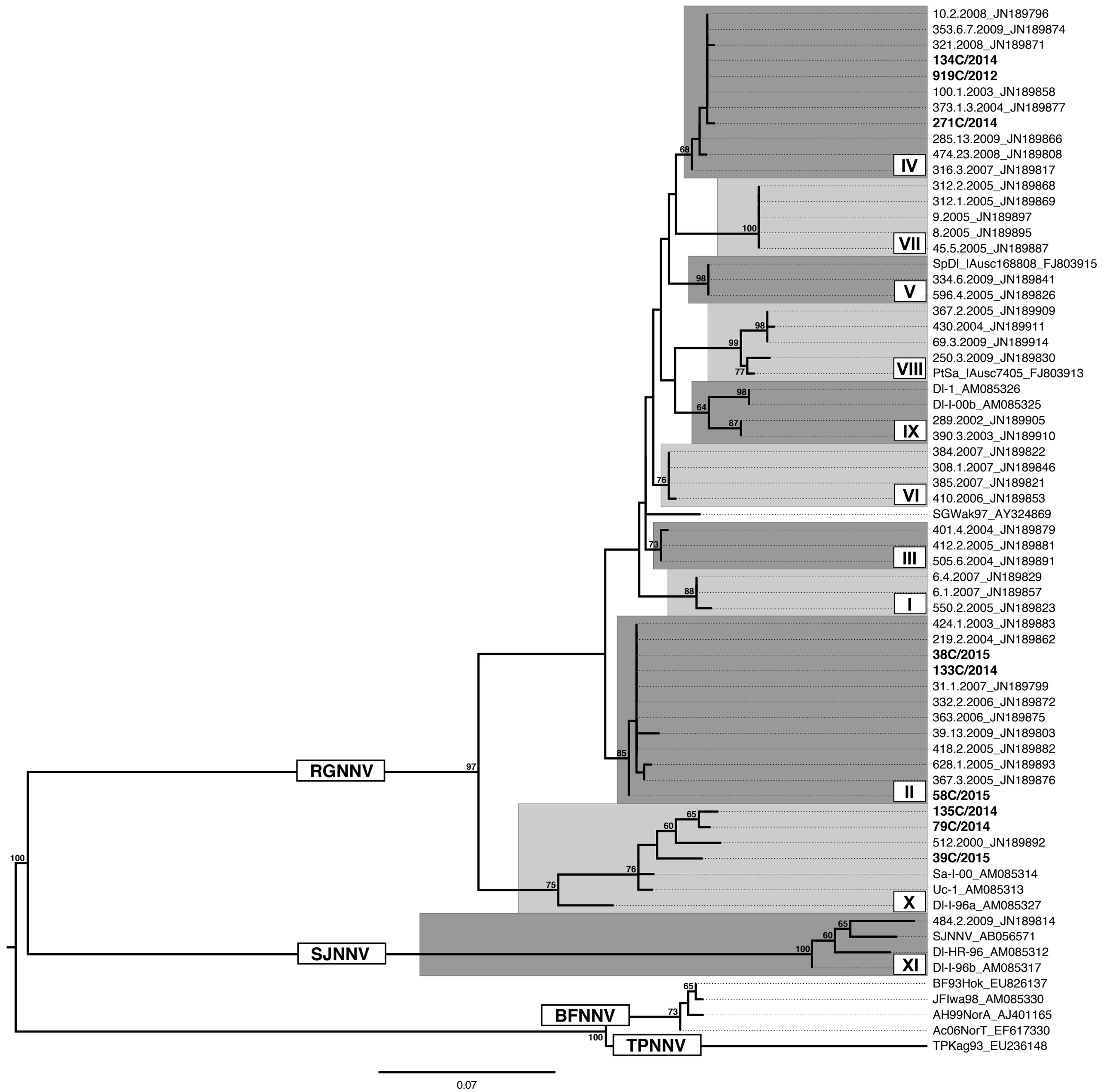
460 Figure legends

461

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.

468

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.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SJNNV
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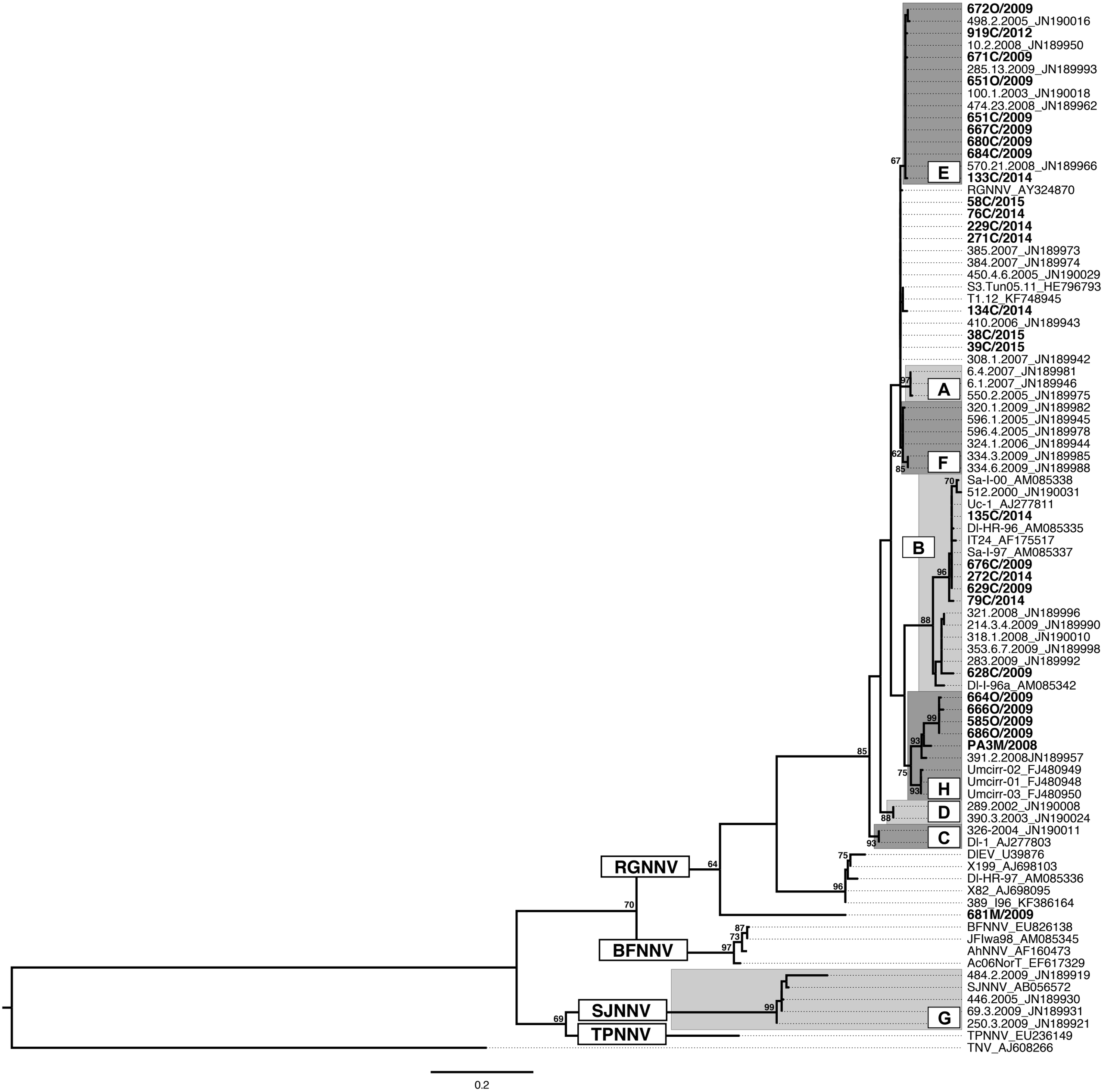
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272C/2014
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