ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs

This is the final peer-reviewed author’s accepted manuscript (postprint) of the following publication:

Published Version:
Volpe, E., Grodzki, M., Panzarin, V., Guercio, A., Purpari, G., Serratore, P., et al. (2018). Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs. JOURNAL OF FISH DISEASES, 41(4), 603-611 [10.1111/jfd.12759].

Availability:
This version is available at: https://hdl.handle.net/11585/620926 since: 2018-03-26

Published:
DOI: http://doi.org/10.1111/jfd.12759

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/).
When citing, please refer to the published version.

(Article begins on next page)

15 January 2025

This is the final peer-reviewed accepted manuscript of:

Volpe, E.; Grodzki, M.; Panzarin, V.; Guercio, A.; Purpari, G.; Serratore, P.; Ciulli, S. (2018). Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs. Journal of Fish Diseases, 41, 603–611. https://doi.org/10.1111/jfd.12759

The final published version is available online at: **https://onlinelibrary.wiley.com/doi/10.1111/jfd.12759**

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/)

When citing, please refer to the published version.

Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs

Running title: Phylogenetic analysis of betanodaviruses

Enrico Volpe[1], Marco Grodzki[1,a], Valentina Panzarin[2], Annalisa Guercio[3], Giuseppa Purpari[3], Patrizia Serratore[1], Sara Ciulli[1]*

[1]Department of Veterinary Medical Sciences, University of Bologna, Cesenatico, Forlì-Cesena, Italy

[2]Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy

[3]Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy

[a]Author's present address

GIGA-Molecular Biology of Diseases, Laboratory of Protein Signaling and Interactions

University of Liège, Liège

*Correspondence:

Sara Ciulli, DIMEVET, UNIBO

Viale Vespucci, 2

47042 Cesenatico, FC, Italy

(e-mail: sara.ciulli@unibo.it)

Acknowledgments

The authors thank Francesca Ellero for English language revision of the manuscript. Santina Di Bella (Istituto Zooprofilattico Sperimentale della Sicilia) is acknowledged for providing technical 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.

43

44

45

46

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

49

50

51

52

53 **1. Introduction**

54

55 Betanodaviruses are small ssRNA viruses of the genus *Betanodavirus*, family *Nodaviridae* (Mori et
56 al., 1992; Thiéry et al., 2012) responsible for viral encephalopathy and retinopathy (VER),
57 otherwise known as viral nervous necrosis (VNN), in several fish species worldwide. The
58 Betanodavirus genome consists of two segments named RNA1 (3.1 KB) and RNA2 (1.4 kb),
59 coding for the RNA-dependent RNA polymerase and the coat protein respectively. Moreover,
60 during virus replication, a subgenomic transcript called RNA3 is originated from the 3' terminus of
61 RNA1 (Mori et al., 1992; Sommerset et al., 2004; Iwamoto et al., 2005; Fenner et al., 2006; Thiéry
62 et al., 2012). Based on the phylogenetic analysis of the T4 variable region within the RNA2
63 segment, betanodaviruses have been clustered into four genotypes, currently accepted as official
64 species of this genus: *Striped jack nervous necrosis virus* (SJNNV), *Tiger puffer nervous necrosis*
65 *virus* (TPNNV), *Barfin flounder nervous necrosis virus* (BFNNV) and *Redspotted grouper nervous*
66 *necrosis virus* (RGNNV) (Nishizawa et al. 1997; Thiéry et al., 2012). An additional genotype
67 clustering outside the four established fish nodavirus species, isolated from *Scophthalmus maximus*
68 and named turbot nodavirus (TNV), has yet to be officially classified (Johansen et al., 2004).
69 Although betanodavirus genotyping is mostly based on RNA2 phylogenetic analysis (Nishizawa et
70 al. 1997), the sequencing of RNA1 has added further information by showing the presence of
71 reassortant strains (Olveira et al., 2009; Toffolo et al., 2007). As a matter of fact, the presence of
72 reassortant betanodaviruses SJNNV/RGNNV had already been described in sea bass
73 (*Dicentrarchus labrax*) from Italy and Croatia, in the form of a genetic variant containing the RNA1
74 segment from the SJNNV genotype and the RNA2 molecule from the RGNNV-type (Toffolo et al.,
75 2007). A new reassortant betanodavirus in the form of a RGNNV/SJNNV genetic variant was later
76 detected in sea bream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*) and common sole
77 (*Solea solea*) farmed in Portugal, Spain and Italy (Olveira 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 *Opistobranchia* (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 μM of each primer, 0.3 μl Superscript III/Platinum Taq enzyme
133 mix and 3 μl RNA in 15 μl 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 MgCl2, 0.25 μM
139 of each primer, 1.25 units of Platinum Taq DNA polymerase, nuclease free water and 1 μl of 1:100-
140 diluted PCR product from the RT-PCR analysis in 25 μl 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'-CACTACCGTGTTGCTG-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).

156 RNA1 (n = 9) and RNA2 (n = 28) nucleotide sequences were aligned and compared with
157 betanodavirus sequences previously obtained from isolates detected in farmed and wild finfish, as
158 well as with betanodavirus reference strains available in GenBank (www.ncbi.nlm.nih.gov) using
159 Clustal W implemented in the BioEdit software (http://bioedit.software.informer.com/). More
160 specifically, we used a representative selection of betanodavirus sequences from southern Europe
161 characterised during a previous molecular epidemiology survey (Panzarin et al., 2012). To describe
162 the phylogenetic relationships among the betanodavirus strains detected in bivalve molluscs and
163 finfish, the maximum likelihood (ML) method available in the PhyML program version 3.1
164 (Guindon et al., 2010) was used. The analysis incorporates the general time-reversible (GTR) model
165 of nucleotide substitution with a gamma distributed rates across sites (four rate categories, $\Gamma 4$) and
166 uses a SPR branch-swapping search procedure (Darriba et al., 2012). One thousand bootstrap
167 replicates were performed to assess the robustness of individual nodes, and only values ≥ 60% were
168 considered significant. Phylogenetic trees were visualized with the FigTree v1.4 software
169 (http://tree.bio.ed.ac.uk/software/figtree/).
170 Amino acid sequences were predicted by the BioEdit software
171 (http://bioedit.software.informer.com/). The percentage of pairwise nucleotide and amino acid
172 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.

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

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

298 **References**

299 Baeck, G. W., Gomez, D. K., Oh, K. S., Kim, J. H., Choresca, C. H. & Park, S. C. (2007).

300 Detection of piscine nodaviruses from apparently healthy wild marine fish in Korea. *Bulletin of the*

301 *European Association of Fish Pathologists*, 27, 116–122.

302 Barker, D. E., MacKinnon, A. M., Boston, L., Burt, M. D. B., Cone, D. K., Speare, D. J., Griffiths,

303 S., Cook, M., Ritchie, R. & Olivier, G. (2002). First report of piscine nodavirus infecting wild

304 winter flounder Pleuronectes americanus in Passamaquoddy Bay, New Brunswick, Canada.

305 *Diseases of Aquatic Organisms,* 49, 99–105. doi:10.3354/dao049099

306 Ciulli, S., Gallardi, D., Scagliarini, A., Battilani, M., Hedrick, R. P. & Prosperi, S. (2006).

307 Temperature-dependency of *Betanodavirus* infection in SSN-1 cell line. *Diseases of Aquatic*

308 *Organisms*, 68, 261–265. doi: 10.3354/dao068261

309 Ciulli, S., Galletti, E., Grodzki, M., Alessi, A., Battilani, M. & Prosperi, S. (2007a). Isolation and
310 Genetic Characterization of *Betanodavirus* from Wild Marine Fish from the Adriatic Sea.
311 *Veterinary Research Communications*, 31, Suppl. 1, 221–224. doi:10.1007/s11259-007-0010-y
312 Ciulli, S., Galletti, E., Grodzki, M., Alessi, A., Battilani, M., Scagliarini, A. & Prosperi, S. (2007b).
313 Spread of B*etanodavirus* in wild marine fish from Adriatic Sea: use of cell culture isolation or
314 nested RT-PCR? In EAFP (Ed.), *Abstract book, 13th International EAFP Conference on "Diseases*
315 *of fish and shellfish"* (p125). Split: Dalmacijapapir publishing house.
316 Ciulli, S., Grodzki, M., Bignami, G., Serratore, P. & Prosperi, S. (2010). Molecular detection and
317 genetic analysis of Betanodaviruses in bivalve mollusks. *Journal of Biotechnology*, 150, S4. doi:
318 10.1016/j.jbiotec.2010.08.026
319 Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012). jModelTest2: more models, new
320 heuristics and parallel computing. *Nature Methods,* 9,772. doi: 10.1038/nmeth.2109.
321 Doan, Q. K., Vandeputte, M., Chatain, B., Morin, T. & Allal, F. (2017). Viral encephalopathy and
322 retinopathy in aquaculture: a review. *Journal of Fish Diseases*, 40, 717–742. doi: 10.1111/jfd.12541
323 Fenner, B. J., Thiagarajan, R., Chua, H. K. & Kwang J. (2006). Betanodavirus B2 is an RNA
324 interference antagonist that facilitates intracellular viral RNA accumulation. *Journal of Virology*,
325 80, 85–94. doi: 10.1128/JVI.80.1.85-94.2006
326 Fichi, G., Cardetti, G., Perrucci, S., Vanni, A., Cersini, A., Lenzi, C., De Wolf, T., Fronte, B.,
327 Guarducci, M. & Susini, F. (2015). Skin lesion-associated pathogens from *Octopus vulgaris*: first
328 detection of *Photobacterium swingsii*, *Lactococcus garvieae* and betanodavirus. *Diseases of aquatic*
329 *organisms*, 115, 147–156. doi: 10.3354/dao02877
330 Gomez, D. K., Sato, J., Mushiake, K., Isshiki, T., Okinaka, Y. & Nakai, T. (2004). PCR-based
331 detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *Journal of*
332 *Fish Diseases,* 27, 603–608. doi: 10.1111/j.1365-2761.2004.00577.x

333 Gomez, D. K., Lim, D. J., Baeck, G. W., Youn, H. J., Shin, N. S., Youn, H. Y., Hwang, C. Y., Park,
334 J. H. & Park, S. C. (2006). Detection of betanodaviruses in apparently healthy aquarium fishes and
335 invertebrates. *Journal of Veterinary Science* 7, 369–374. doi: 10.4142/jvs.2006.7.4.369
336 Gomez, D. K., Baeck, G. W., Kim, J. H., Choresca, C. H. Jr. & Park, S. C. (2008a). Molecular
337 detection of Betanodavirus in wild marine fish populations in Korea. *Journal of Veterinary*
338 *Diagnostic Investigation*, 20, 38–44. doi: 10.1177/104063870802000107
339 Gomez, D. K., Baeck, G. W., Kim, J. H., Choresca, C. H. Jr. & Park, S. C. (2008b). Molecular
340 detection of betanodaviruses from apparently healthy wild marine invertebrates. *Journal of*
341 *Invertebrate Pathology* **97,** 197–202. doi: 10.1016/j.jip.2007.10.012
342 Gomez, D. K., Baeck, G. W., Kim, J. H., Choresca, C. H. J. & Park, S.C. (2008c). Genetic analysis
343 of betanodaviruses in subclinically infected aquarium fish and invertebrates. *Current Microbiology*,
344 56, 499–504. doi: 10.1007/s00284-008-9116-x
345 Gomez, D. K., Matsuoka, S., Mori, K., Okinaka, Y., Park, S.C. & Nakai, T. (2009). Genetic
346 analysis and pathogenicity of *Betanodavirus* isolated from wild redspotted grouper Epinephelus
347 akaara with clinical signs. *Archives of Virology,* 154, 343–346. doi: 10.1007/s00705-008-0305-5
348 Gomez, D. K., Mori, K., Okinaka, Y., Nakai, T. & Park, S. C. (2010). Trash fish can be a source of
349 betanodaviruses for cultured marine fish. *Aquaculture*, 302, 158–163. doi:
350 10.1016/j.aquaculture.2010.02.033
351 Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New
352 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of
353 PhyML3.0. *Systematic biology,* 59, 307–321. doi: 10.1093/sysbio/syq010
354 Iwamoto, T., Mise, K., Taked, A., Okinaka, Y., Mori, F., Arimoto, M., Okuno, T. & Nakai, T.
355 (2005). Characterization of Striped jack nervous necrosis virus subgenomic RNA3 and biological
356 activities of its encoded protein B2. *Journal of General Virology,* 86, 2807– 2816. doi:
357 10.1099/vir.0.80902-0

358 Johansen, R., Sommerset, I., Tørud, B., Korsnes, K., Hjortaas, M. J., Nilsen, F., Nerland, A. H. &
359 Dannevig, B. H. (2004). Characterization of nodavirus and viral encephalopathy and retinopathy in
360 farmed turbot Scophthalmus maximus (L). *Journal of Fish Diseases*, 27, 591–601. doi:
361 10.1111/j.1365-2761.2004.00581.x
362 Liu, X. D., Huang, J. N., Weng, S. P., Hu, X.Q., Chen, W. J., Qin, Z. D., Dong, X. X., Liu, X. L.,
363 Zhou, Y., Asim, M., Wang, W.M., He, J.G. & Lin, L. (2015). Infections of nervous necrosis virus in
364 wild and cage-reared marine fish from South China Sea with unexpected wide host ranges. *Journal*
365 *of Fish Diseases*, 38, 533-540. doi: 10.1111/jfd.12265
366 Mori, K., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K. & Furusawa, I. (1992). Properties of a
367 new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous
368 necrosis. *Virology*, 187, 368–371.
369 Nishizawa, T., K. Mori, T. Nakai, Furusawa, I. & Muroga, K. (1994). Polymerase chain reaction
370 (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Diseases of Aquatic*
371 *Organisms,* 18,103–107.
372 Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T. & Muroga, K. (1997). Genomic classification of
373 fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Applied and*
374 *environmental microbiology,* 63, 1633–1636.
375 Olveira, J. G., Souto, S., Dopazo, C. P., Thiéry, R., Barja, J. L. & Bandín, I. (2009). Comparative
376 analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed
377 fish species provides evidence for genetic reassortment. *The Journal of general virology*, 90, 2940–
378 2951. doi: 10.1099/vir.0.013912-0
379 Panzarin, V., Fusaro, A., Monne, I., Cappellozza, E., Patarnello, P., Bovo, G., Capua, I., Holmes, E.
380 C. & Cattoli, G. (2012). Molecular epidemiology and evolutionary dynamics of betanodavirus in
381 southern Europe. *Infection, Genetics and Evolution,* 12, 63–70. doi: 10.1016/j.meegid.2011.10.007
382 Serratore, P., Ciulli, S., Piano, A. & Cariani, A. (2014). Criticism of the purification process of
383 bivalve shellfish. Literature review and our industrial research experiences. In R.M. Hay (Ed.),

384 *Shellfish human consumption, health implications and conservation concerns (*pp. 1-50). New York:
385 Nova Science Publishers, Inc.
386 Sommerset, I. & Nerland, A. H. (2004). Complete sequence of RNA1 and subgenomic RNA3 of
387 Atlantic halibut nodavirus (AHNV). *Diseases of Aquatic Organisms,* 58, 117–125. doi:
388 10.3354/dao058117
389 Thiéry, R., Johnson, K. L., Nakai, T., Schneemann, A., Bonami, J. R. & Lightner, D. V., (2012).
390 Family Nodaviridae. In: A.M. Q. King, M. J. Adams, E. B. Carstens, E. J. Lefkowitz (Eds.), *Virus*
391 *Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses* (pp. 1061-1067).
392 California: Elsevier Academic Press.
393 Toffolo, V., Negrisolo, E., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., Dalla Valle, L.,
394 (2007). Phylogeny of betanodaviruses and molecular evolution of their RNA polymerase and coat
395 proteins. *Molecular Phylogenetics and Evolution*, 43, 298–308. doi: 10.1016/j.ympev.2006.08.003
396 Vendramin, N., Patarnello, P., Toffan, A., Panzarin, V.,Cappellozza, E., Tedesco, P. & Cattoli, G.
397 (2013). Viral Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a
398 threat for wild endangered species. *BMC Veterinary Research,* 9, 20. doi: 10.1186/1746-6148-9-20
399 Volpe, E., Pagnini, N., Serratore, P., & Ciulli, S. (2017). Fate of Redspotted grouper nervous
400 necrosis virus (RGNNV) in experimentally challenged Manila clam *Ruditapes philippinarum*.
401 *Diseases of Aquatic Organisms,* 125, 53–61. doi: 10.3354/dao03139
402
403
404
405
406
407
408

409 **Table 1.** Details of betanodavirus strains retrieved from bivalve molluscs and used for phylogenetic
410 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

433 nd: not determinated

434 **Table 2.** Comparisons of nucleotide and amino acid sequences of a RNA2 fragment, which include
435 the variable region of betanodaviruses detected in bivalve molluscs with reference to betanodavirus
436 strains (RGNNV: AY324870; BFNNV: EU826138; SJNNV: AB056572; TPNNV: EU236149;
437 Thiery et al., 2012). Percentage of pairwise nucleotide and amino acid similarity are shown.
438
439

	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

440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459

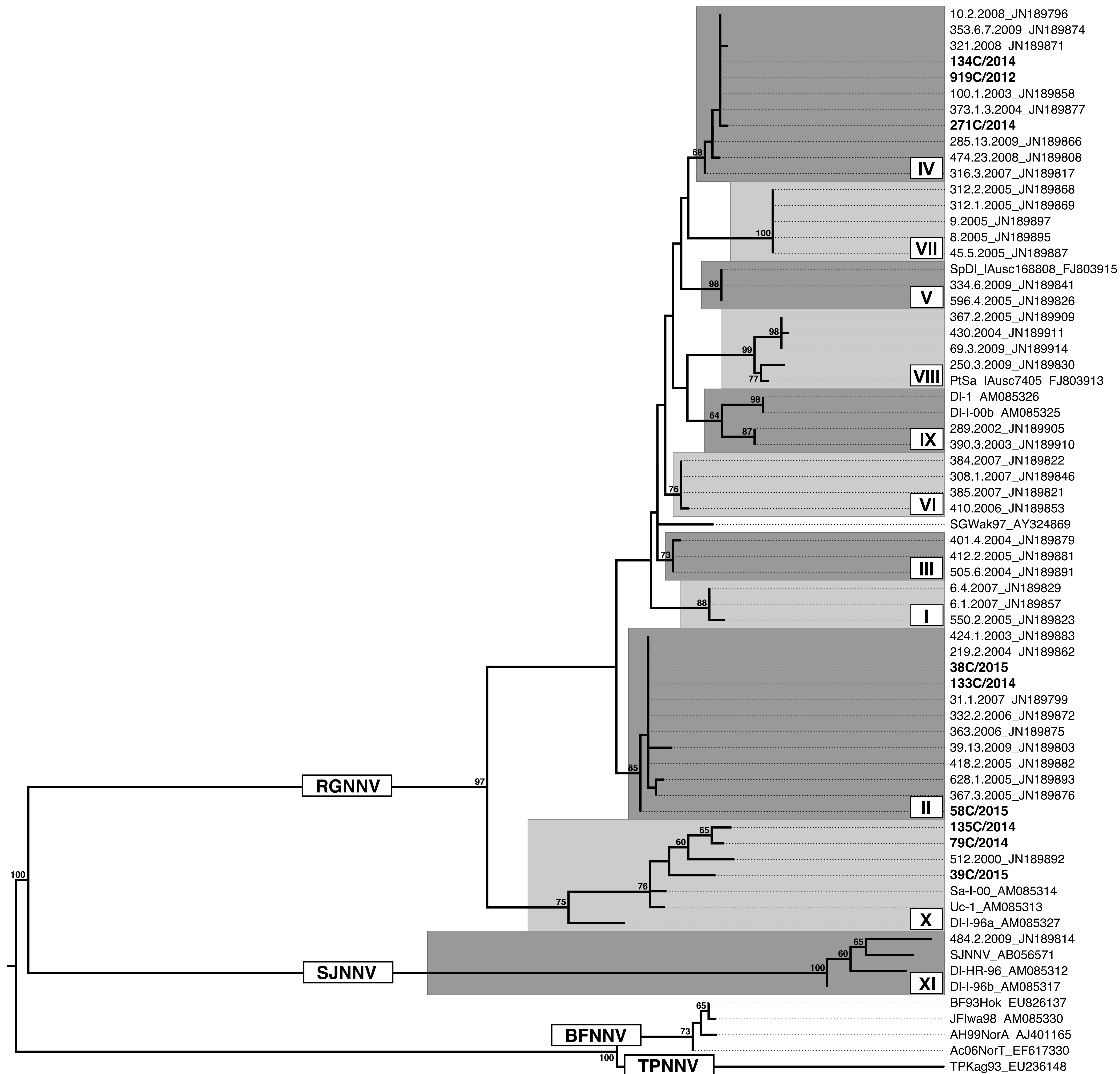
460 Figure legends

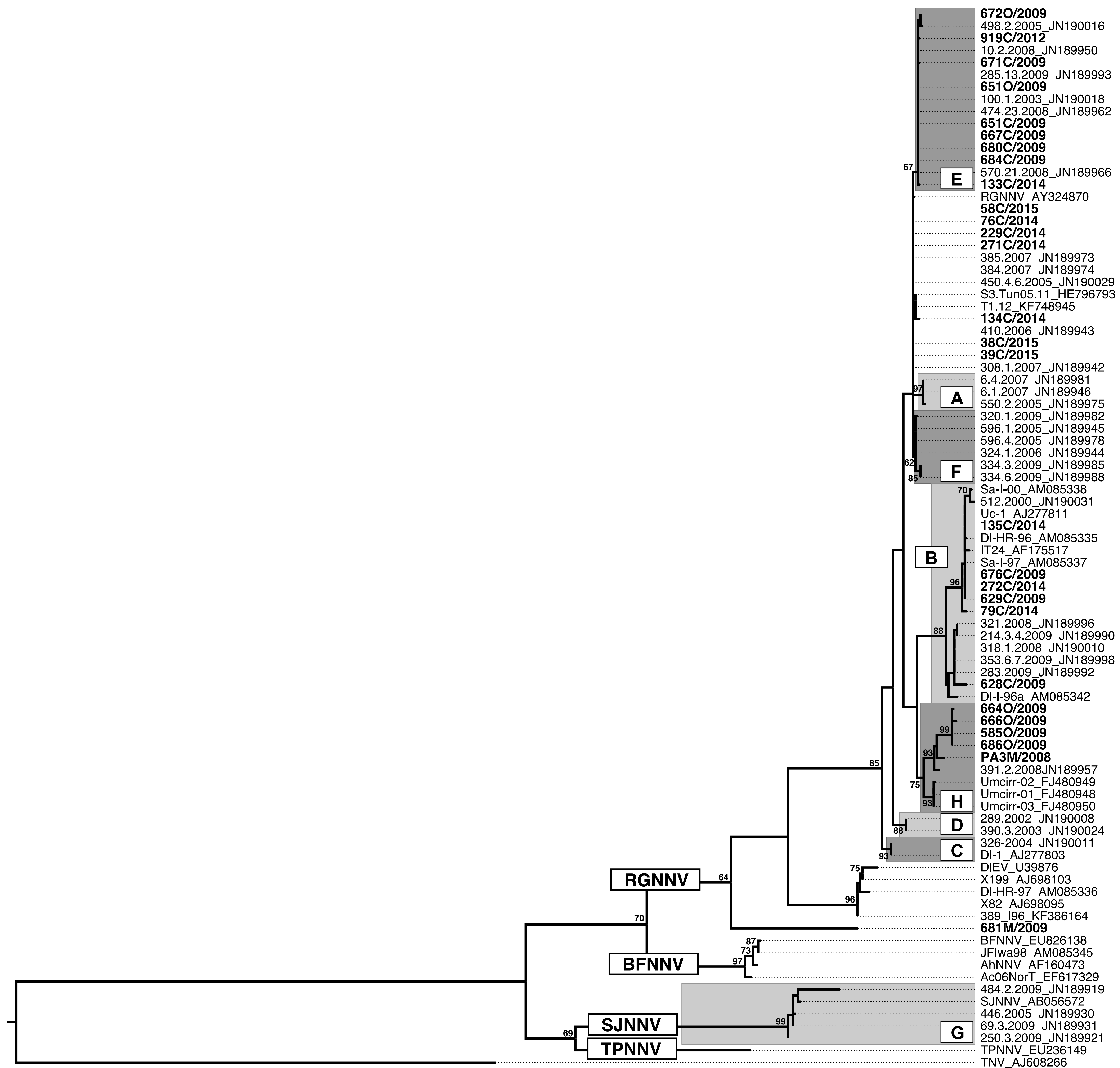
461

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.


10.2.2008_JN189796
353.6.7.2009_JN189874
321.2008_JN189871
134C/2014
919C/2012
100.1.2003_JN189858
373.1.3.2004_JN189877
271C/2014
285.13.2009_JN189866
474.23.2008_JN189808
316.3.2007_JN189817
312.2.2005_JN189868
312.1.2005_JN189869
9.2005_JN189897
8.2005_JN189895
45.5.2005_JN189887
SpDl_IAusc168808_FJ803915
334.6.2009_JN189841
596.4.2005_JN189826
367.2.2005_JN189909
430.2004_JN189911
69.3.2009_JN189914
250.3.2009_JN189830
PtSa_IAusc7405_FJ803913
Dl-1_AM085326
Dl-I-00b_AM085325
289.2002_JN189905
390.3.2003_JN189910
384.2007_JN189822
308.1.2007_JN189846
385.2007_JN189821
410.2006_JN189853
SGWak97_AY324869
401.4.2004_JN189879
412.2.2005_JN189881
505.6.2004_JN189891
6.4.2007_JN189829
6.1.2007_JN189857
550.2.2005_JN189823
424.1.2003_JN189883
219.2.2004_JN189862
38C/2015
133C/2014
31.1.2007_JN189799
332.2.2006_JN189872
363.2006_JN189875
39.13.2009_JN189803
418.2.2005_JN189882
628.1.2005_JN189893
367.3.2005_JN189876
58C/2015
135C/2014
79C/2014
512.2000_JN189892
39C/2015
Sa-I-00_AM085314
Uc-1_AM085313
Dl-I-96a_AM085327
484.2.2009_JN189814
SJNNV_AB056571
Dl-HR-96_AM085312
Dl-I-96b_AM085317
BF93Hok_EU826137
JFIwa98_AM085330
AH99NorA_AJ401165
Ac06NorT_EF617330
TPKag93_EU236148
IV
VII
V
VIII
IX
VI
III
I
II
X
XI
RGNNV
SJNNV
BFNNV
TPNNV
0.07


672O/2009
498.2.2005_JN190016
919C/2012
10.2.2008_JN189950
671C/2009
285.13.2009_JN189993
651O/2009
100.1.2003_JN190018
474.23.2008_JN189962
651C/2009
667C/2009
680C/2009
684C/2009
570.21.2008_JN189966
133C/2014
E
67
RGNNV_AY324870
58C/2015
76C/2014
229C/2014
271C/2014
385.2007_JN189973
384.2007_JN189974
450.4.6.2005_JN190029
S3.Tun05.11_HE796793
T1.12_KF748945
134C/2014
410.2006_JN189943
38C/2015
39C/2015
308.1.2007_JN189942
6.4.2007_JN189981
6.1.2007_JN189946
550.2.2005_JN189975
A
97
320.1.2009_JN189982
596.1.2005_JN189945
596.4.2005_JN189978
324.1.2006_JN189944
334.3.2009_JN189985
334.6.2009_JN189988
F
62
85
Sa-I-00_AM085338
512.2000_JN190031
Uc-1_AJ277811
135C/2014
DI-HR-96_AM085335
IT24_AF175517
Sa-I-97_AM085337
676C/2009
272C/2014
629C/2009
79C/2014
321.2008_JN189996
214.3.4.2009_JN189990
318.1.2008_JN190010
353.6.7.2009_JN189998
283.2009_JN189992
628C/2009
DI-I-96a_AM085342
B
70
96
88
664O/2009
666O/2009
585O/2009
686O/2009
PA3M/2008
391.2.2008JN189957
Umcirr-02_FJ480949
Umcirr-01_FJ480948
Umcirr-03_FJ480950
H
99
93
75
93
289.2002_JN190008
390.3.2003_JN190024
D
88
326-2004_JN190011
DI-1_AJ277803
C
93
85
DIEV_U39876
X199_AJ698103
DI-HR-97_AM085336
X82_AJ698095
389_I96_KF386164
75
96
681M/2009
RGNNV
64
70
BFNNV_EU826138
JFIwa98_AM085345
AhNNV_AF160473
Ac06NorT_EF617329
BFNNV
87
73
97
484.2.2009_JN189919
SJNNV_AB056572
446.2005_JN189930
69.3.2009_JN189931
250.3.2009_JN189921
G
99
SJNNV
69
TPNNV
TPNNV_EU236149
TNV_AJ608266
0.2