Risk assessment of wild fish as environmental sources of red sea bream iridovirus (RSIV) outbreaks in aquaculture

Assessing the risk of wild fish around aquaculture environment as a source of red sea bream iridovirus (RSIV) outbreak

Yasuhiko Kawato[1,*], Kaori Mizuno[2], Shogo Harakawa[2], Yuzo Takada[1], Yusaku Yoshihara[3], Hidemasa Kawakami[2], Takafumi Ito[1]

[1] Pathology Division, Nansei Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Mie 519-0193, Japan

[2] Ehime Fisheries Research Center, Ehime 798-0087, Japan

[3] Ainan Town Fisheries Division, Ehime 798-4292, Japan

Running Head: Assessing RSIV risk in wild fish

[*] Address correspondence to Yasuhiko Kawato, kawato_yasuhiko86@fra.go.jp

ABSTRACT

Red sea bream iridovirus (RSIV) causes substantial economic damage to aquaculture. In the present study, RSIV in wild fish in an aquaculture environment was surveyed to evaluate the risk of wild fish being an infection source for RSIV outbreaks in cultured fish. In total, 1102 wild fish, consisting of 44 species, were captured from two aquaculture areas in western Japan using fishing, gill nets, and fishing baskets between 2019 and 2022. Eleven fish from seven species were confirmed to harbor the RSIV genome using a probe-based real-time PCR assay. The mean viral load of the RSIV-positive wild fish was $10^{1.1 \pm 0.4}$ copies/mg DNA, which was significantly lower than that of seemingly healthy red sea bream (*Pagrus major*) in a net pen during an RSIV outbreak ($10^{3.3 \pm 1.5}$ copies/mg DNA) that occurred in 2021. Sequencing analysis of a partial region of the major capsid protein gene demonstrated that the RSIV genome detected in the wild fish was identical to that of the diseased fish in a fish farm located in the same area where the wild fish was captured. Based on the diagnostic records of RSIV in the sampled area, the RSIV-infected wild fish appeared during RSIV outbreak or after the ceasing of the disease in cultured fish, suggesting that RSIV detected in wild fish was derived from the RSIV outbreak in cultured fish. Therefore, wild fish populations around aquaculture environments may not be a

significant risk factor for RSIV outbreaks in cultured fish.

Keywords: red sea bream iridovirus, RSIV, *Megalocytivirus*, wild fish, *Pagrus major*

1. INTRODUCTION

Red sea bream iridovirus (RSIV), a lethal pathogen in various fish species, belongs to the genus *Megalocytivirus* in the family *Iridoviridae* (Jancovich et al. 2012). The virus has a double-stranded DNA genome approximately 110 kbp in length. The viral particles are 160–180 nm in diameter and include an envelope-like structure around the icosahedral virion capsid (Kurita & Nakajima 2012; Kawato et al. 2020). Megalocytiviruses have been divided into two species by the International Committee on Taxonomy of Viruses (ICTV): *infectious spleen and kidney necrosis virus* (*ISKNV*), to which RSIV belongs, and *scale drop disease virus* (*SDDV*), which has recently been registered as a new species in the genus (Chinchar et al. 2017). Based on phylogenetic analysis of the major capsid protein (MCP) and ATPase genes, *ISKNV* species can be mainly classified into three genogroups, each with two clades: the RSIV genogroup originating from marine fish, the ISKNV genogroup originating from both marine and freshwater fish, and the turbot reddish body iridovirus (TRBIV) genogroup, which is mainly reported in marine flatfish (Kurita & Nakajima 2012, Go et al. 2016, Koda et al. 2018).

RSIV was first reported in Japan in 1990 in cultured red sea bream (*Pagrus major*), from which the viral name was derived (Inouye et al. 1992). The affected fish show signs of

lethargy, have pale gills and an enlarged spleen, and numerous enlarged cells are observed in the visceral organs, such as the spleen, heart, and kidney (Inouye et al. 1992). The viral infection has caused significant economic damage to more than 30 mariculture fish species every summer season in Japan for more than 30 years (Matsuoka et al. 1996; Kawakami & Nakajima, 2002). After the emergence of RSIV in Japan, similar viral infections caused by megalocytiviruses have been reported in Asian countries (He et al. 2000; He et al. 2001; Jung & Oh, 2000; Do et al. 2004; Shi et al. 2004; Shi et al. 2010). Furthermore, the geographical distribution of megalocytiviruses has spread to Europe, the Americas, and Africa since the 2010s in both aquaculture fish for human consumption (Subramaniam et al. 2016; Lopez-Porras et al., 2018; Ramírez-Paredes 2020; Figueiredo et al. 2022) and ornamental fish (Jung-Schroers et al. 2016; Bermúdez et al. 2018). Because of its significant impact on the aquaculture industry, RSIV infection caused by the *ISKNV* species has been designated as a notifiable disease by the World Organization for Animal Health (WOAH, 2023).

Seasonal infection cycles and viral carriers during off-season RSIV outbreaks in aquaculture environments remain unclear. Since the viral infection in Japan routinely occurs every summer season and ceases in winter, there could be potential virus carriers or vectors in aquaculture environments, such as surviving fish (Ito et al. 2013, Ito et al. 2014), latently

73 infected fish in which viral replication is suppressed at lower temperatures (Jun et al. 2009,
74 Oh et al. 2014), wild fish (Wang et al. 2007), and bivalves near fish farms (Jin et al. 2014).
75 Recently, we suggested that asymptomatically infected broodstock could be an infection
76 source for an outbreak event on a fish farm (Kawato et al. 2021).
77 Assessing the risk of environmental water (seawater) and wild fish for RSIV outbreak are
78 required to implement appropriate biosecurity measures in fish farms as seawater and wild
79 fish can move freely among net pens in the mariculture system. Our latest research has shown
80 that the seawater contributes less than expected to the transmission of RSIV among fish farms
81 because the transmission via seawater was highly associated with the distance between the
82 net pens (Kawato et al. 2023). Therefore, in this study, we assessed the wild fish around
83 aquaculture environment as a potential risk of RSIV outbreaks in fish farms. We surveyed
84 wild fish in two fish farming areas where RSIV outbreaks have routinely occurred between
85 2019 and 2022. The risk of wild fish for RSIV outbreak is discussed based on the results of
86 surveillance accompanied by the status of the RSIV epidemic in the area where the wild fish
87 were captured.
88
89 **2. MATERIALS AND METHODS**

2.1 Fish samples

Wild fish were collected from two bay areas (A and B) located in western Japan, where more than one million cultured fish, such as red sea bream and Japanese amberjack (*Seriola quinqueradiata*), were reared (Fig. 1). A total of 1,102 fish were captured using fishing, gill nets, and fishing baskets between 2019 and 2021 in area A, and between 2020 and 2022 in area B. Wild fish samples consisted of 8 orders, 29 families, and 44 species (Table 1). For comparison with cultured fish, 8 dead and 30 seemingly healthy fish were collected from a red sea bream fish farm located in area B during the RSIV outbreak in 2021. Fish samples were kept on ice in a vessel, and spleens were collected on the same day. The spleen samples were stored at −80°C until DNA extraction.

2.2 Epidemiological information on RSIV disease

Information on RSIV epidemics in each area was obtained from diagnostic records inspected by two diagnostic laboratories for fish diseases managed by local authorities. RSIV infection of each case was confirmed by observation of enlarged cells which is a typical sign of RSIV-infected fish using stamp smear of spleen or kidney by Giemsa stain (Inouye et al. 1992). In areas A and B, the diagnostic records were summarized by the

number of fish farms and individual net pens, respectively, owing to differences in the recording systems of the diagnostic laboratories. There were huge outbreaks of RSIV in 2021 in areas A and B because most fish farms introduced RSIV-infected juveniles.

2.3 DNA extraction and real-time PCR

Total DNA was extracted from spleen samples using the QIAamp DNA Mini Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. The extracted DNA was examined using a real-time PCR targeting the MCP gene of RSIV (Kawato et al. 2021) using the RSIV-MCP186F (5′-CGG CCA GGA GTT TAG TGT GAC T-3′) and RSIV-MCP288R primers (5′-GCT GTT CTC CTT GCT GGA CG-3′) and the RSIV-MCP239P probe (5′-FAM-TGT GGC TGC GTG TTA AGA TCC CCT CCA-BHQ1-3′).

Briefly, 20 μL of the reaction mixture, consisting of 2 μL of the DNA template, 10 μL of THUNDERBIRD Probe qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan), and a final concentration of 200 nM for each primer and probe, was added to each well. For copy number calculation, a serial 10-fold dilution of a plasmid (pGEM-Easy vector; Promega Corporation, Madison, WI, USA) containing the target region of real-time PCR was used to draw a standard curve. Real-time PCR was performed using a LightCycler 96 Instrument

124 (Roche Diagnostics K.K., Tokyo, Japan) or CFX96 Touch Real-Time PCR Detection
125 System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplification thermal
126 profile consisted of preincubation at 95°C for 1 min followed by 45 cycles of denaturation
127 and annealing, which were conducted at 95°C for 10 s and 60°C for 30 s, respectively. The
128 samples were tested in duplicate.
129
130 2.4 Sequencing analysis
131 A partial region of the MCP gene of RSIV from wild and cultured fish (dead and seemingly
132 healthy) from the net pen during the RSIV outbreak was sequenced. PCR primers, RSIV
133 MCP-216F (5′-GGG TGG CGA CTA CCT CAT TA -3′) and RSIV MCP-793R (5′-CAA
134 TGA GCA TGC TAC GGC TA-3′) were used for the 1st PCR to amplify the partial region
135 of the MCP gene (578 bp). PCR amplification was performed using KOD One PCR Master
136 Mix -Blue- (TOYOBO Co., Ltd., Osaka, Japan). The amplification thermal profile
137 consisted of 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and
138 extension at 68°C for 30 s. Then, a nested PCR was performed using the amplicon of the 1st
139 PCR, which was diluted 100 times with distilled water. PCR primers for the nested PCR
140 were RSIV MCP-251F (5′-TTA AGA TCC CCT CCA TCA CG-3′) and RSIV MCP-764R

(5′-ACA ACC TCA CGC TCC TCA CT -3′). The amplification thermal profile consisted of one denaturation cycle of 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 68°C for 1 s. The 514 bp amplicon was purified using Illustra ExoProStar (Global Life Sciences Technologies Japan K.K., Tokyo, Japan) and directly sequenced using a DNA sequencing service (Fasmac Co., Ltd., Kanagawa, Japan). The nucleotide sequences, except for the primer region of the partial MCP gene (474 bp), were submitted to the DNA Data Bank of Japan (DDBJ) under accession number LC779498-LC779503. Phylogenetic tree analysis was performed with the partial MCP nucleotide sequences obtained from wild and cultured fish (dead and seemingly healthy) during the outbreak, and 17 megalocytiviruses collected from the NCBI GenBank database. The MCP sequence of scale drop disease virus (SDDV) was selected as an outgroup. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 10.2.6 (Kumar et al. 2018) using the neighbor-joining method with default parameters and 1000 bootstrap replicates.

2.5 Statistical analysis

For the copy number of RSIV genome among dead fish, asymptomatic infected fish, and wild fish, assumptions of the normality and variances were tested first using Shapiro-Wilk test and Bartlett test, respectively. Because the normality was confirmed but the variances were not homogeneous, the data sets were analyzed by nonparametric test using Welch's one-way ANOVA followed by pairwise Welch's T-test with the Bonferroni correction as the post hoc test. The detection rate of the RSIV genome between sampling areas or among cultured and wild fish was analyzed using Fisher's exact test, and the p-value was adjusted using the Bonferroni method. The mean days from the date of wild fish sampling to that of first RSIV outbreak in each year was compared between area A and B using Welch's T-test. Statistical significance was set at $P < 0.05$. The analyses were performed using R version 4.2.1 using packages "multcomp" and "RVAideMemoire."

3. RESULTS

3.1 Detection of RSIV genome from wild fish around aquaculture environment

RSIV genome was detected in 11 wild fish from 7 species among 1102 wild fish consisting of 8 orders, 29 families, and 44 species captured around two aquaculture environment (Tables 1 and 2). The RSIV-detected species were limited in Perciformes fish although

174 there were 248 samples (22.5%) derived from non-Perciformes fish. The RSIV detection
175 frequencies in areas A (1.2%) and B (0.9%) were not significantly different (Table 1). Most
176 of RSIV-detected wild fish were confirmed in 2021 when huge outbreaks of RSIV were
177 reported due to introducing RSIV-infected juveniles as seedlings in many fish farms (Table
178 2, Fig 2). No RSIV-positive wild fish was confirmed in area A in 2020 when no RSIV
179 epidemic was recorded (Fig. 2A). On the other hand, RSIV was not detected from the wild
180 fish captured in area B in 2020 although RSIV outbreaks were confirmed (Fig. 2B). All the
181 RSIV-detected wild fish were confirmed after RSIV outbreak in cultured fish in both areas
182 where the wild fish were captured (Table 2, Fig. 2). The mean days between the date of
183 sampling and that of first RSIV outbreak in each year was 85.4 ± 44.3 days and it was
184 not significantly different between area A (94.8 ± 32.1 days) and B (74.0 ±57.6 days).
185
186 3.2 Comparison between cultured fish and wild fish
187 The viral genome was detected in all dead fish and in 19 of 30 seemingly healthy fish
188 collected from cultured red sea bream during the RSIV outbreak. The detection frequencies
189 of RSIV among dead, seemingly healthy, and wild fish were 100%, 63.3%, and 1.0%,
190 respectively, and that of wild fish was significantly low. Although both RSIV-detected wild

191 fish and cultured fish had no clinical signs, the mean viral load in the wild fish ($10^{1.1\pm0.4}$
192 copies/mg DNA) was significantly lower than that of the seemingly healthy fish ($10^{3.5\pm1.6}$,
193 copies/mg DNA) (Fig. 3).
194 The partial nucleotide sequences of the MCP gene, except for the primer region (474 bp)
195 detected from eight positive samples in the wild fish, were completely identical. Although
196 no PCR product was obtained using nested PCR in the three positive samples from
197 Japanese jack mackerel (*Trachurus japonicus*), a partial RSIV sequence was confirmed by
198 sequencing the real-time PCR amplicons. The nucleotide sequences detected in wild fish
199 were identical between dead and seemingly healthy fish during the RSIV outbreak (Fig. 4).
200 Phylogenetic tree analysis indicated that all nucleotide sequences detected in the wild fish
201 belonged to the RSIV genogroup Clade 2 (Fig. 4).
202

203 **4. DISCUSSION**

204 The present study demonstrated the frequency and intensity of RSIV infection in wild fish
205 captured in an aquaculture environment using real-time PCR assay. Many wild fish have
206 been considered potential carriers of RSIV epidemics in fish farms because RSIV has a
207 broad host range for more than 30 species of marine fish (Matsuoka et al. 1996; Kawakami

208 & Nakajima 2002). Indeed, ISKNV, another genogroup of RSIV in the megalocytiviruses,
209 was detected in 9.1% (n = 1118) of wild fish using nested PCRs (Wang et al. 2007). In
210 contrast, the 1.0% (n = 1102) detection rate in wild fish observed in this study was
211 significantly lower than that in the previous study despite a similar sample size. RSIV-
212 positive wild fish were limited to seven species within Perciformes fish in this study,
213 whereas the ISKNV-positive wild fish are confirmed in 36 species across six orders by
214 Wang et al. (2007). Although the potential differences in the sensitivity and specificity of
215 the assays used in both studies should be considered, the detection frequency in wild fish
216 could vary according to geographical location and aquaculture conditions.
217 Many wild fish especially Perciformes fish could be potential susceptible species for RSIV.
218 Among RSIV-detected wild fish, 4 species *(Trachurus japonicus*, *P. major*, *Girella*
219 *punctata*, and *Epinephelus awoara*) are reported to be susceptible to RSIV (Matsuoka et al.
220 1996; Kawakami & Nakajima, 2002). On the other hand, to the best of our knowledge, the
221 other RSIV-detected wild fish (*Evynnis tumifrons*, *Prionurus scalprum*, and *Sebastiscus*
222 *marmoratus*) classified into Perciformes is the first confirmed case for RSIV infection.
223 Considering the extremely broad host range of RSIV (Kawato et al. 2017), it could be a
224 potential risk of RSIV outbreak to start aquaculture of new fish species especially

225 Perciformes fish.

226 The RSIV load in wild fish was extremely low, suggesting that RSIV transmission from
227 wild fish to cultured fish is rare. In the asymptomatically RSIV-infected fish during the
228 RSIV outbreak in the fish farm, a range of viral loads between $10^{1.1}$ and $10^{5.9}$ copies/mg
229 DNA was observed. A similar situation was observed in a challenge test when RSIV was
230 exposed to red sea bream at 10^{6} copies/L continuously for 3 days, which mimics a field
231 outbreak (Kawato et al. 2023). In the challenge test, RSIV shedding into the rearing water
232 from infected fish was confirmed, which could be a source for expanding the disease in fish
233 farms. Therefore, the variation in viral load during the asymptomatic stage indicates the
234 progression of the RSIV outbreak in the population. In contrast, the viral load of the wild
235 fish did not exceed $10^{2.0}$ copies/mg DNA, suggesting that the RSIV infection did not
236 progress in the infected wild fish for individuals and populations, at least when the fish
237 were captured. Furthermore, the low viral load in wild fish indicates that few viruses could
238 be shed into environmental water from RSIV-infected wild fish. In addition, wild fish do
239 not have the opportunity to come into direct contact with cultured fish because they are
240 separated by a net pen. It is unlikely that RSIV-infected wild fish remain close to the net
241 pen of cultured fish and release a large number of viral particles to transmit the virus

because the wild fish can freely move among net pens in aquaculture environment. Indeed, our previous study monitoring the RSIV load in seawater in aquaculture environments demonstrated that there were only 7 samples exceeding $10^{4.0}$ copies/L of the RSIV load in seawater among 308 seawater samples between 2019 and 2022. All the high viral load samples in seawater were associated with RSIV outbreak in fish farms (Kawato et al. 2023). In addition, our infection model indicates that RSIV-contained seawater with less than $10^{3.0}$ copies/L can be ignored as an infection source for RSIV outbreak. Thus, the RSIV-infected wild fish which were low viral load and prevalence, and physically separated by the cultured fish, was not considered to be the infection source for RSIV outbreak. There could be infection sources for RSIV outbreak in fish farms other than RSIV-infected wild fish. For example, the diseases are confirmed in 2020 in area B although no RSIV was detected from the wild fish (Fig. 2). Our previous study suggests that asymptomatically RSIV-infected broodstock could be the infection source for RSIV outbreak in aquaculture environment as high viral load was detected from seawater at the net pen of seemingly healthy broodstock of red sea bream followed by confirmation of RSIV outbreak in the juveniles nearby the broodstock net pen in 10 days (Kawato et al. 2021). Similar situations could be occurred in area B in 2020 because the cases were mainly confirmed in fish farms

where different fish ages were reared in a limited area. In contrast, RSIV outbreaks in 2021 in areas A and B have been confirmed to be due to introduction of RSIV-infected juveniles possibly transmitted from the asymptomatically RSIV-infected broodstock (Kawato et al. 2023). Therefore, there is no evidence that the RSIV-infected wild fish was the source of RSIV outbreak so far.

The RSIV detected in wild fish was presumed to have been derived from fish farms where the RSIV outbreak occurred. The target region for sequencing analysis contained single-nucleotide polymorphisms (SNPs) that could distinguish among several RSIV isolates (unpublished data). For example, the SNPs suggested that the RSIV genome of the wild fish in this study was different from that of RSIV RS-17 (accession no. LC605053) isolated from red sea bream during an outbreak in a different prefecture in 2017 (Kawato et al. 2021) but was identical to that in the cultured red sea bream in area A in 2021. Furthermore, the comparison with the diagnostic records of RSIV indicated that RSIV-infected wild fish appeared during or after the outbreak. The mean days between the date of wild fish sampling and that of first RSIV outbreak in cultured fish was not different between area A and B suggesting that similar situation was occurred in both areas. It should also be noted that wild fish did not harbor RSIV when no RSIV epidemics were confirmed in area A in

2020. These results suggest that RSIV was transmitted from the cultured fish to the wild fish rather than the RSIV-infected wild fish was the source of the disease outbreak in fish farms. Furthermore, the RSIV infection was hardly considered to be circulating among wild fish as there were cohorts of wild fish that RSIV was not detected in 2020.

The limited risk of RSIV-infected wild fish transmitting RSIV to cultured fish suggests that the transmission of RSIV between fish farms could be partly due to the movement of equipment and humans associated with fish farms. Our previous study suggested that the transmission of RSIV via environmental water is highly associated with the distance between net pens; hence, environmental water is not always an infection source for the transmission of RSIV between fish farms (Kawato et al. 2023). Another factor for transmission between fish farms that cannot be controlled in semi-open aquaculture systems is wild fish. However, our surveillance of wild fish suggests that RSIV-infected wild fish contribute little to the transmission of RSIV to cultured fish. Therefore, biosecurity management, such as the disinfection of equipment associated with fish farms, could be effective, even in semi-open aquaculture systems, where wild fish and environmental water can freely move. Further studies are needed to identify the transmission routes of RSIV to fish farms.

CONFLICT OF INTEREST

The authors declare no conflicting interests.

ACKNOWLEDGEMENT

This study was supported by the Regulatory Research Projects for Food Safety, Animal Health, and Plant Protection (JPJ00867.19190702) funded by the Ministry of Agriculture, Forestry, and Fisheries of Japan. A part of this research was supported by the Food Safety and Consumer Affairs Bureau, the Ministry of Agriculture, Forestry, and Fisheries of Japan.

References

Bermúdez R, Losada AP, de Azevedo AM, Guerra-Varela J, Pérez-Fernández D, Sánchez L, Padrós F, Nowak B, Quiroga MI. (2018) First description of a natural infection with spleen and kidney necrosis virus in zebrafish. J Fish Dis 41:1283–1294

Chinchar VG, Hick P, Ince IA, Jancovich JK, Marschang R, Qin Q, Subramaniam K, Waltzek TB, Whittington R, Williams T, Zhang Q-Y, ICTV Report Consortium. (2017)

ICTV virus taxonomy profile: Iridoviridae. J Gen Virol 98:890–891

Do JW, Moon CH, Kim HJ, Ko MS, Kim SB, Son JH, Kim JS, An EJ, Kim MK, Lee SK, Han MS, Cha SJ, Park MS, Park MA, Kim YC, Kim JW, Park JW. (2004) Complete genomic DNA sequence of rock bream iridovirus. Virology 325:351−363

Figueiredo HCP, Tavares GC, Dorella FA, Rosa JCC, Marcelino SAC, Pierezan F, Pereira FL (2022) First report of infectious spleen and kidney necrosis virus in Nile tilapia in Brazil. Transbound Emerg Dis 69:3008−3015

Go J, Waltzek TB, Subramaniam K, Yun SC and others (2016) Detection of infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) from archival ornamental fish samples. Dis Aquat Org 122:105−123

He JG, Weng SP, Zeng K, Huang ZJ, Chan SM (2000) Systemic disease caused by an iridovirus-like agent in cultured mandarinfish, *Siniperca chuatsi* (Basilewsky), in China. J Fish Dis 23:219−222

He JG, Deng M, Weng SP, Li Z, Zhou SY, Long QX, Wang XZ, Chan SM (2001) Complete genome analysis of the mandarin fish infectious spleen and kidney necrosis iridovirus. Virology 291:126−139

Inouye K, Yamano K, Maeno Y, Nakajima K, Matsuoka M, Wada Y, Sorimachi M (1992)

Iridovirus infection of cultured red sea bream, *Pagrus major*. Fish Pathol 27:19−27

Ito T, Yoshiura Y, Kamaishi T, Yoshida K, Nakajima K (2013) Prevalence of red sea bream iridovirus among organs of Japanese amberjack (*Seriola quinqueradiata*) exposed to cultured red sea bream iridovirus. J Gen Virol 94:2094−2101

Ito T, Kawato Y, Yoshiura Y, Kamaishi T, Yoshida K, Nakajima K (2014) Potential infectivity of the virus re-isolated from surviving Japanese amberjack (*Seriola quinqueradiata*) after experimental infection with red sea bream iridovirus. B Eur Assoc Fish Pat 34:17−24

Jancovich JK, Chinchar VG, Hyatt A, Miyazaki T, Williams T, Zhang QY (2012) Iridoviridae, p. 207−210. *In* King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (ed), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Academic Press, London.

Jin JW, Kim KI, Kim JK, Park NG, Jeong HD (2014) Dynamics of megalocytivirus transmission between bivalve molluscs and rock bream *Oplegnathus fasciatus*. Aquaculture 428−429:29−34

Jung SJ, Oh MJ (2000) Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the

344 Korean peninsula. J Fish Dis 23:223−226
345 Jung-Schroers V, Adamek M, Wohlsein P, Wolter J, Wedekind H, Steinhagen D (2016) First
346 outbreak of an infection with infectious spleen and kidney necrosis virus (ISKNV) in
347 ornamental fish in Germany. Dis Aquat Org 119:239–244
348 Jun LJ, Jeong JB, Kim JH, Nam JH, Shin KW, Kim JK, Kang JC, Jeong HD (2009)
349 Influence of temperature shifts on the onset and development of red sea bream iridoviral
350 disease in rock bream *Oplegnathus fasciatus*. Dis Aquat Organ 84: 201−208
351 Kawakami H, Nakajima K (2002) Cultured fish species affected by red sea bream iridoviral
352 disease from 1996 to 2000. Fish Pathol 37:45−47
353 Kawato Y, Mekata T, Inada M, Ito T (2021) Application of environmental DNA for
354 monitoring red sea bream iridovirus at a fish farm. Microbiol Spectr 9:e0079621
355 Kawato Y, Mohr PG, Crane MSJ, Williams LM, Neave MJ, Cummins DM, Dearnley M,
356 Crameri S, Holmes C, Hoad J, Moody NJG (2020) Isolation and characterisation of an
357 ISKNV-genotype megalocytivirus from imported angelfish *Pterophyllum scalare*. Dis
358 Aquat Organ 140:129–141
359 Kawato Y, Subramaniam K, Nakajima K, Waltzek T, Whittington R (2017) Iridoviral
360 diseases: red sea bream iridovirus and white sturgeon iridovirus. In: Woo PTK, Cipriano

RC (eds) Fish viruses and bacteria: Pathobiology and Protection. CABI Publishing, Wallingford, p 147–159

Kawato Y, Takada Y, Mizuno K, Harakawa S, Yoshihara Y, Nakagawa Y, Kurobe T, Kawakami H, Ito T (2023) Assessing the transmission risk of red sea bream iridovirus (RSIV) in environmental water: Insights from fish farms and experimental settings. Microbiol Spectr 11:e0156723

Koda SA, Subramaniam K, Francis-Floyd R, Yanong RP, Frasca S Jr, Groff JM, Popov VL, Fraser WA, Yan A, Mohan S, Waltzek TB (2018) Phylogenomic characterization of two novel members of the genus *Megalocytivirus* from archived ornamental fish samples. Dis Aquat Org 130:11−24

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547-1549

Kurita J, Nakajima K (2012) Megalocytiviruses. Viruses 4:521−538.

Lopez-Porras A, Morales JA, Alvarado G, Koda SA, Camus A, Subramaniam K, Waltzek TB, Soto E (2018) Red seabream iridovirus associated with cultured Florida pompano *Trachinotus carolinus* mortality in Central America. Dis Aquat Org 130:109−115

Matsuoka S, Inouye K, Nakajima K (1996) Cultured fish species affected by red sea bream

iridoviral disease from 1991 to 1995. Fish Pathol 31:233−234

Oh SY, Oh MJ, Nishizawa T (2014) Potential for a live red seabream iridovirus (RSIV) vaccine in rock bream *Oplegnathus fasciatus* at a low rearing temperature. Vaccine. 32:363−368

Ramírez-Paredes JG, Paley RK, Hunt W, Feist SW, Stone DM, Field TR, Haydon DJ, Ziddah PA, Nkansa M, Guilder J, Gray J, Duodu S, Pecku EK, Awuni JA, Wallis TS, Verner-Jeffreys DW (2021) First detection of infectious spleen and kidney necrosis virus (ISKNV) associated with massive mortalities in farmed tilapia in Africa. Transbound Emerg Dis 68:1550-1563

Shi CY, Wang YG, Yang SL, Huang J, Wang QY (2004) The first report of an iridovirus-like agent infection in farmed turbot, *Scophthalmus maximus*, in China. Aquaculture 236:11−25

Shi CY, Jia KT, Yang B, Huang J (2010) Complete genome sequence of a Megalocytivirus (family Iridoviridae) associated with turbot mortality in China. Virol J 7:159

Subramaniam K, Gotesman M, Smith CE, Steckler NK, Kelley KL, Groff JM, Waltzek TB (2016) Megalocytivirus infection in cultured Nile tilapia *Oreochromis niloticus*. Dis Aquat Organ 119:253−258

Wang YQ, Lu L, Weng SP, Huang JN, Chan SM, He JG (2007) Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus-like (ISKNV-like) virus. Arch Virol 152:763−773

WOAH (World Organisation for Animal Health) (2023) Infection with red sea bream iridovirus. In: Aquatic Animal Health Code. https://www.woah.org/fileadmin/Home/eng/Health_standards/aahc/current/chapitre_rsbid.pdf

403

404

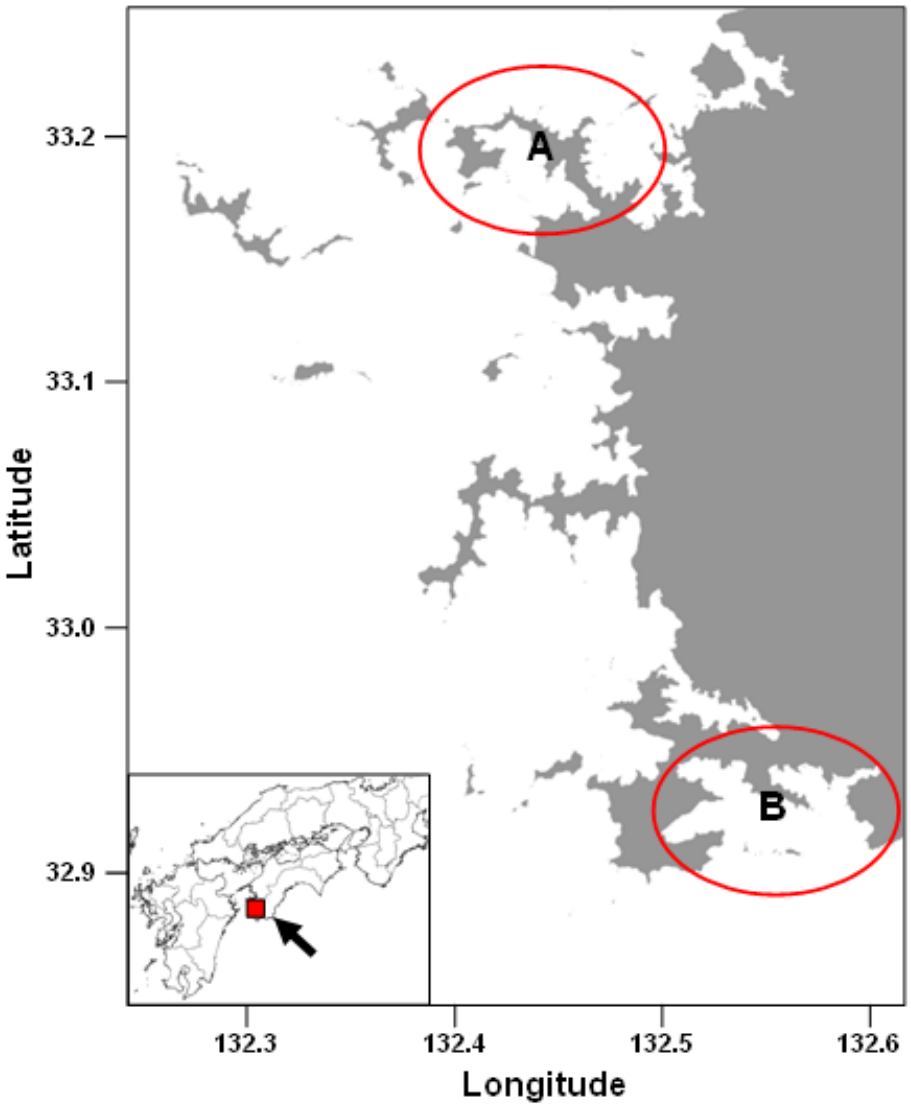

Fig. 1. Sampling area of wild fish. Fish were captured using fishing, gill net, and fishing basket between 2019 and 2022 in area A and B. The map was created by QGIS 3.34.3 using a GIS data obtained from Geospatial Information Authority of Japan. Red filled square with an arrow in wide-area map indicates the sampling area in Ehime prefecture in Japan.

405

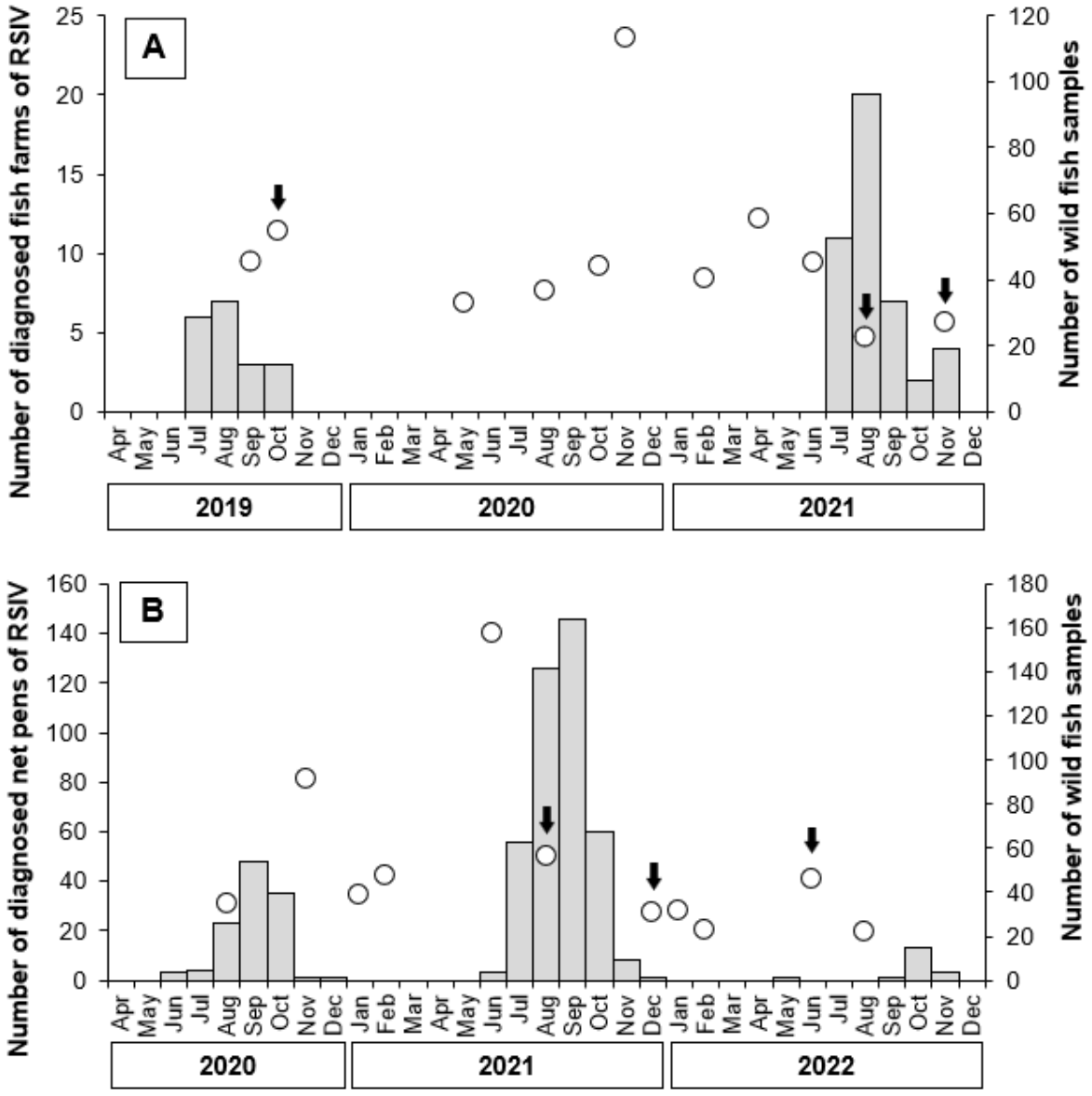

Fig. 2. Relationship between RSIV outbreak in cultured fish and appearance of RSIV-infected wild fish. Number of diagnosed cases of RSIV in cultured fish in the area A and B each year are shown by grey-filled histogram. White-filled circles indicate months when wild fish were captured and number of the wild fish sample. Black arrows indicate months when RSIV was detected from the wild fish.

406

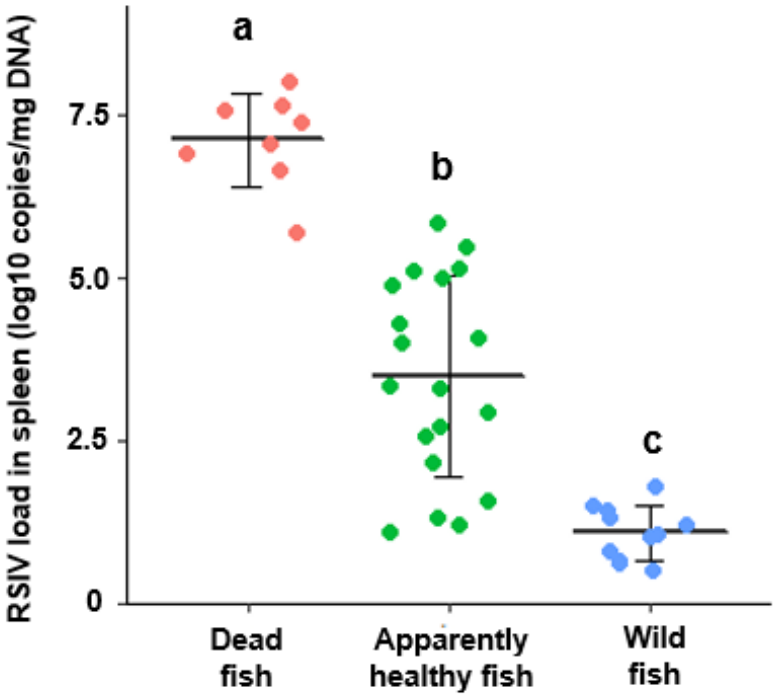

Fig. 3. Distribution of RSIV load among dead fish, apparently healthy fish, and wild fish from which RSIV was detected. The dead fish and apparently healthy fish were collected from a net pen of red sea bream where RSIV outbreaks. Alphabets above each chart indicate statistical significance of mean viral loads which was set at $P < 0.05$.

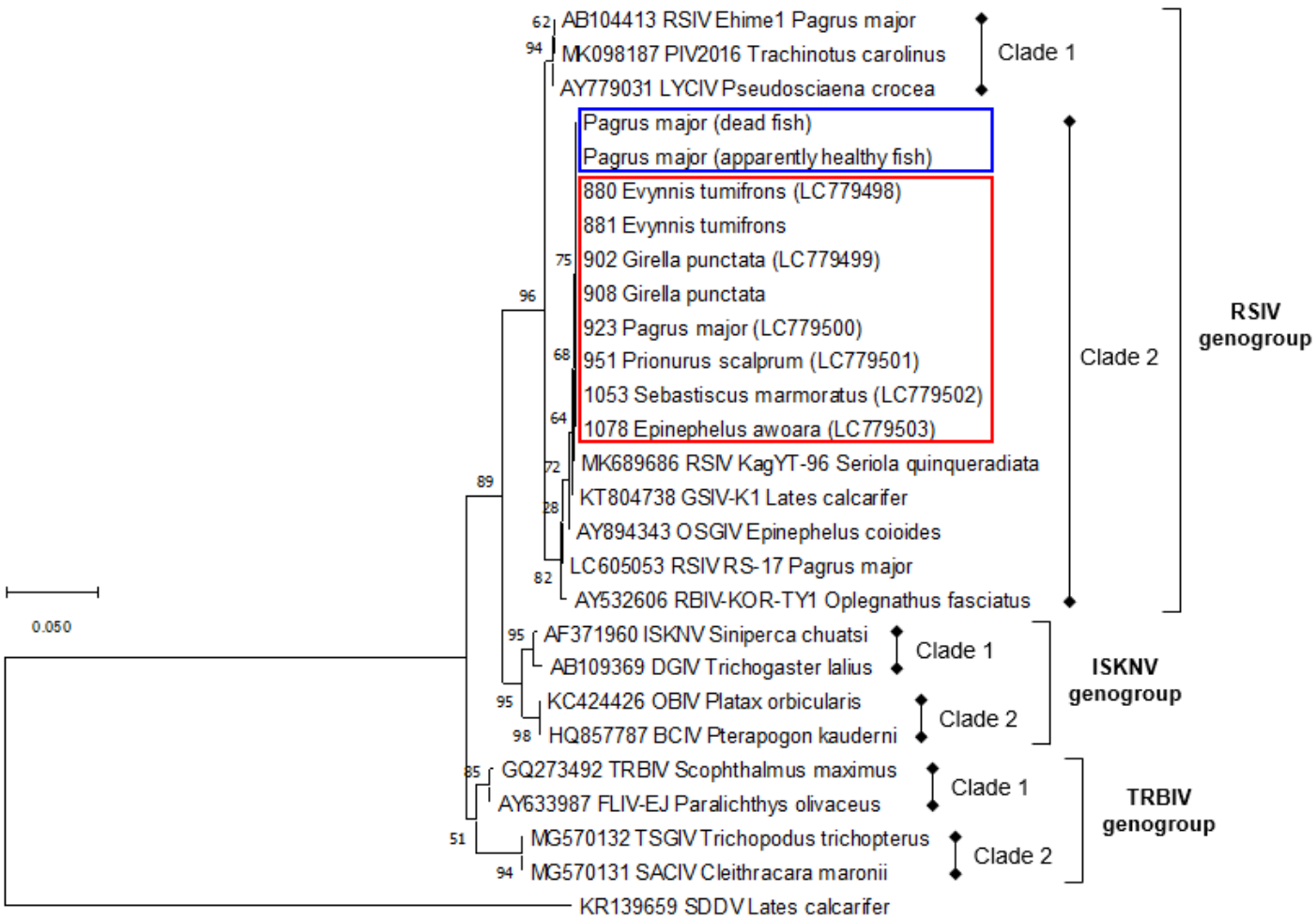

Fig. 4. Phylogenetic tree of megalocytiviruses based on the major capsid protein (MCP) gene sequenced in this study and registered sequences. The tree was constructed by the Neighbor-Joining method which was rooted from the MCP sequence of scale drop disease virus (SDDV). The numbers shown next to the branch nodes indicate the percentage of bootstrap support for each node calculated from 1,000 replicates. The scale bar indicates the number of nucleic acid substitutions per site. Nucleotide sequences highlighted by the red frame are the wild fish samples with sample number and fish species. Blue frame indicates the dead fish and apparently healthy fish collected from a net pen during RSIV outbreak in 2021. The other sequences are shown together with the Genbank accession number, name of viral isolate, and host fish species. Description of each genogroup and clade is adapted from Go et al. (2016) and Koda et al. (2018).

407

408 Table 1. Surveillance of red sea bream iridovirus (RSIV) in wild fish around aquaculture environment

Order	Family	Species	Positive fish/Examined fish		
			Area A	Area B	Total
Anguilliformes	Muraenesocidae	*Muraenesox cinereus*	0/10	-[*1]	0/10
Aulopiformes	Synodontidae	*Saurida sp.*	0/69	-	0/69
Clupeiformes	Dussumieriidae	*Etrumeus micropus*	0/21	-	0/21
	Engraulidae	*Engraulis japonicus*	0/1	-	0/1
Lophiiformes	Lophiidae	*Lophiomus setigerus*	0/1	-	0/1
Mugiliformes	Mugilidae	*Mugil cephalus*	0/1	-	0/1
Perciformes	Acanthuridae	*Prionurus scalprum*	-	1/5	1/5
	Apogonidae	*Ostorhinchus semilineatus*	0/1	0/12	0/13
		Pristicon trimaculatus	0/1	-	0/1
	Carangidae	*Carangoides equula*	0/3	0/25	0/28
		Decapterus maruadsi	0/25	0/44	0/69
		Seriola quinqueradiata	0/26	0/65	0/91
		Trachurus japonicus	3/106	0/24	3/130
	Chaetodontidae	*Roa modesta*	0/1	-	0/1
	Haemulidae	*Parapristipoma trilineatum*	-	0/3	0/3
		Plectorhinchus cinctus	-	0/3	0/3
		Diagramma pictum	-	0/1	0/1
	Kyphosidae	*Oplegnathus punctatus*	-	0/4	0/4
		Oplegnathus fasciatus	-	0/9	0/9
		Girella punctata	-	2/138	2/138
	Labridae	*Choerodon azurio*	0/4	0/2	0/6
		Pseudolabrus sieboldi	0/2	-	0/2
	Latridae	*Goniistius zonatus*	-	0/1	0/1
	Lethrinidae	*Lethrinus haematopterus*	-	0/3	0/3
	Lutjanidae	*Lutjanus kasmira*	0/1	0/1	0/2
	Mullidae	*Parupeneus chrysopleuron*	-	0/2	0/2
		Upeneus japonicus	-	0/25	0/25
	Nemipteridae	*Parascolopsis eriomma*	0/8	-	0/8
	Pomacanthidae	*Chaetodontoplus septentrionalis*	0/2	-	0/2
	Pomacentridae	*Chromis notata*	0/1	0/2	0/3
	Pteroinae	*Pterois lunulata*	0/5	-	0/5
	Scombridae	*Scomber japonicus*	0/2	-	0/2
	Scorpaenidae	*Sebastiscus marmoratus*	0/10	1/40	1/50
	Sebastidae	*Sebastes sp.*	0/1	-	0/1

	Serranidae	*Epinephelus awoara*	-	1/2	1/2
		Epinephelus areolatus	-	0/8	0/8
	Sparidae	*Evynnis tumifrons*	2/109	0/8	2/117
		Pagrus major	1/75	0/12	1/87
		Rhabdosargus sarba	-	0/31	0/31
	Triglidae	*Lepidotrigla microptera*	0/1	-	0/1
Tetraodontiformes	Monacanthidae	*Aluterus monoceros*	-	0/4	0/4
		Stephanolepis cirrhifer	0/30	0/98	0/128
		Thamnaconus modestus	0/1	0/11	0/12
Zeiformes	Zeidae	*Zeus faber*	0/1	-	0/1
Total positive fish/Total examined fish (%)			6/519 (1.2)	5/583 (0.9)	11/1102 (1.0)

409 [*1] -: no sample

410

411

Table 2. Information of wild fish from which RSIV was detected.

Sample No.	Area	Sampling date	Days after outbreak*1	Captured method	Species	BW (g)	RSIV copies /mg DNA	Accession number*2
74	A	16-Oct-19	107	Fishing	*Trachurus japonicus*	111	2.8E+01	-
76	A	16-Oct-19	107	Fishing	*Trachurus japonicus*	140	4.6E+00	-
77	A	16-Oct-19	107	Fishing	*Trachurus japonicus*	130	2.1E+01	-
880	A	30-Aug-21	56	Gill net	*Evynnis tumifrons*	380	3.2E+00	LC779498.1
881	A	30-Aug-21	56	Gill net	*Evynnis tumifrons*	280	4.0E+00	-
923	A	18-Nov-21	136	Gill net	*Pagrus major*	100	1.1E+01	LC779500.1
902	B	31-Aug-21	74	Fishing	*Girella punctata*	840	1.7E+01	LC779499.1
908	B	31-Aug-21	74	Fishing	*Girella punctata*	440	6.3E+00	-
951	B	3-Dec-21	168	Fishing	*Prionurus scalprum*	500	1.2E+01	LC779501.1
1053	B	23-Jun-22	27	Fishing	*Sebastiscus marmoratus*	285	6.3E+01	LC779502.1
1078	B	23-Jun-22	27	Fishing	*Epinephelus awoara*	650	3.2E+01	LC779503.1

*1 Days between the date of sampling and that of first RSIV outbreak in cultured fish in the area where the wild fish was captured in each year.

*2 Partial sequence of the major capsid protein gene of RSIV determined by amplicon sequence of the nested PCR.