JFMSE, 30(6), pp. 2024~2035, 2018.
수산해양교육연구, 제30권 제6호, 통권96호, 2018.

www.ksfme.or.kr
https://doi.org/10.13000/JFMSE.2018.12.30.6.2024

Molecular Characterization of Megalocytiviruses from Diseased Fishes in Korean Aquatic Farms from 2013 to 2017

Young-Chul KIM* · Jee-Youn HWANG** · Hae-Ryeon JEON* · Da-Won LEE* · Jung-Soo SEO** ·
Kwang-Il KIM** · Mun-Gyeong KWON*** · Bo-Young JEE*** · Seong-Don HWANG**†

*National Institute of Fisheries Science (research worker) · **†National Institute of Fisheries Science (researcher)
· ***National Institute of Fisheries Science (senior researcher)

2013년~2017년 국내 양식장 어류에서 검출된 megalocytiviruses의 분자생물학적 특성

김영철* · 황지연** · 전해련* · 이다원* · 서정수** · 김광일** ·
권문경*** · 지보영*** · 황성돈**†
*국립수산과학원(연구원) · **†국립수산과학원(연구사) · ***국립수산과학원(연구관)

Abstract

We identified structural and non-structural gene regions encoding major capsid protein (MCP) and DNA polymerase (DPOL) of megalocytiviruses collected from infected cultured fishes in RBIVD outbreak farms in 2013-2017 in Korea. With the two PCRs using 1-F/1-R and 4-F/4-R primer sets of the Manual of Diagnosis Tests for Aquatic Animals of the World Organization for Animal Health (OIE), amplicons were generated from the spleen and kidney tissue from approxmately ~30 fishes, including rock bream (*Oplegnathus fasciatus*), red sea bream (*Pagrus major*), and rock fish (*Sebastes schlegeli*), from 15 outbreak regions in the aquatic farms of the South Sea and Jeju Island. In phylogenetic analysis, complete MCP and partial DPOL genes belonged to RSIV type-subgroup2. Interestingly, these genes formed a cluster indicating closer relatedness to GSIV-K1, RIE12-1, and RBIV-C1, which were previously isolated from Japan and China, than with RBIV-KOR-TY1 isolated from Korea. However, the nucleotide sequence identities of the MCP and DPOL genes of these viruses were high, at >99.8% and >99.7%, respectively, compared with RBIV-KOR-TY1. Comparisons of nucleotide and amino acid sequences showed minimal differences between the obtained strains in the MCP gene, however, one or two nucleotide sequences substitutions of the DPOL gene were detected in nine strains, including a silent mutation detected in five strains. These findings suggest a slow rate of evolution of megalocytiviruses in this region, but the potential for mutations and new pathogenic strains warrants continuous surveillance.

Key words : Megalocytivirus, RSIV, RBIV, MCP, DNA polymerase, Phylogenetic tree

Ⅰ. Introduction

Members of the family *Iridoviridae* genus *Megalocytivirus* are icosahedral viruses that contain a double-stranded DNA genome (Rimmer et al., 2016; Subramaniam et al., 2012; Whittington et al.,

---

† Corresponding author : 051-720-3043, sdhwang@korea.kr

※ This work was supported by a grant from the National Institute of Fisheries Science (R2018065).

2010). This genus is the major agent causing disease in >50 freshwater and marine fish species, resulting in significant economic losses to the aquaculture industry in East and Southeast Asia (Song et al., 2008; Jung et al., 2017a; Inouye et al., 1992; Shi et al., 2004).

The Iridoviridae comprises the following five genera: *Megalocytivirus, Ranavirus, Lymophocystivirus, Iridovirus*, and *Choloriridovirus* (Chinchar et al., 2005). Megalocytiviruses have been divided into three types based on phylogenetic analysis of the major capsid protein (MCP) gene: the red sea bream iridovirus (RSIV) type cluster, including RSIV subgroup1 and rock bream iridovirus (RBIV) subgroup2, reported as the major epizootic viral strains affecting aquaculture worldwide (Kim et al. 2018, Jung and Oh, 2000; Zhang et al., 2012. 2013); infectious spleen and kidney necrosis virus (ISKNV) type that generally causes diseases in freshwater fish species (He et al. 2000); and turbot reddish body iridovirus (TRBIV) type (Shi et al., 2004), which infects flatfish species.

Megalocytiviruses mainly occur in summer when the water temperature reaches 23°C and above (Jung et al., 2015, 2016, 2017a, 2017b; Jun et al., 2009). Diseased fish show signs of lethargy, exhibit severe anemia and petechiae in the gills, and have enlarged spleens. Histopathology is characterized by development of enlarged cells with basophilic inclusion bodies in the spleen (Do et al., 2004; Do et al., 2005; OIE 2018). In Korea, megalocytiviruses have caused massive damage to aquacultures of various fish species such as rock bream (*Oplegnathus fasciatus*), rockfish (*Sebastes schlegelii*), red sea bream (*Pagrus major*), and olive flounder (*Paralichthys olivaceus*) (Jeong et al., 2003, 2006; Jin et al., 2018; Won et al., 2013; Kim et al., 2018).

Megalocytiviruses are notable for their variability in infecting a broad host range. Genomic analysis of RSIVs can be useful in identifying the introduction of exotic viruses due to the importation of live fish or natural fish migration from the sea of other countries. Thus, gaining a better understanding of the genetic diversity of megalocytiviruses would provide critical information of their biology and evolution to guide strategies for controlling these diseases. To contribute such information, in this study, we investigated the genetic relationships of the MCP and DNA polymerase (DPOL) genes of megalocytiviruses, especially RSIV type-subgroup2, isolated from diseased aquacultured fish in Korea from 2013 to 2017 and compared these findings with previous reports.

Ⅱ. Materials and Methods

1. Samples

Moribund or dead fishes showing typical clinical symptoms of iridoviral disease were collected from 15 domestic aquatic farms by surveillance, disease identification, or an investigation into damage of the National Institute of Fisheries Science (NIFS) and institutions specializing in the disease identification of aquatic organism diseases from 2013 to 2017. The aquatic disease control division of the NIFS confirmed the RBIVD outbreak by verification with additional experiments. Specifically, rock bream was sampled in Tongyeong (four farms), Yeosu (five farms), Wando (one farm), Sacheon (one farm), Namhae (one farm), and Seogwipo of Jeju Island (one farm) and red sea bream and rock fish were sampled from one farm each at Tongyeong <Table 1>. Pooled samples including spleen and kidney tissue from approximately ~30 fishes collected at each farm were used for virus detection.

2. Total nucleic acid extraction and megalocytivirus identification

Total nucleic acids (50 μL) were isolated from 200 μL of the tissue homogenate in phosphate buffered saline (pH 7.4, 1:10 w/v) using a Viral NA Extraction Kit on a SPRI-TE^{TM} Nucleic Acid Extractor (Beckman Coulter Inc., USA) according to the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed in a final volume of 25 μL with 2 μL of DNA template using Takara EX Taq^{TM} (Takara) in a Mastercycler (Eppendorf, Germany). The primer sets 1-F/1-R and 4-F/4-R (Kurita et al. 1998) were used for megalocytivirus detection, and reaction conditions were determined according to the Manual of Diagnostic Tests for Aquatic Animals of the World Organization for Animal Health (OIE, 2018). The PCR conditions for sequence analysis were as follows: pre-denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 s, 58°C for 60s, and 72°C for 60 s; followed by an extension period at 72°C for 5 min. The amplified PCR products were subjected to QIAxcel Advanced System (Qiagen). For identification of megalocytivirus, nucleotide sequences were determined by the ABI 3500xl genetic analyzer (Thermo Fisher Scientific, UK)using the PCR products purified from a PCR Purification Kit (GeneAll, Korea).

<Table 1>. Samples infected with megalocytivirus used in this study

Viral strains	Year of occurence	Geographic origin	Host species
ADC-RSIV2013-24	2013	Yeosu	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2014-35	2014	Seogwipo	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2014-44	2014	Tongyeong	Rock fish *Sebastes schlegelii*
ADC-RSIV2014-46	2014	Tongyeong	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2015-24	2015	Yeosu	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2016-2	2016	Yeosu	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2016-29	2016	Wando	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2016-38	2016	Yeosu	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2016-41	2016	Tongyeong	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2016-56	2016	Tongyeong	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2017-18	2017	Namhae	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2017-22	2017	Sacheon	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2017-27	2017	Tongyeong	Red sea bream *Pagrus major*
ADC-RSIV2017-49	2017	Tongyeong	Rock bream *Oplegnathus fasciatus*
ADC-RSIV2017-68	2017	Yeosu	Rock bream *Oplegnathus fasciatus*

<Table 2>. Primers used in this study

Primer	Sequence (5' to 3')	Expected size (object)	Reference
1-F	CTCAAACACTCTGGCTCATC	570 bp (Detection)	Kurita et al. 1998
1-R	GCACCAACACATCTCCTATC		
4-F	CGGGGGCAATGACGACTACA	568 bp (Detection)	Kurita et al. 1998
4-R	CCGCCTGTGCCTTTTCTGGA		
M1F	GAGAGACCCCAACACGAC	1828 bp (MCP gene analysis)	He et al. 2001
M1R	ACCTGGTGGCTCCAGTGC		
DPO-F	CTTCAGCTTCAGGTTCACGCATGC	1264 bp (DPOL gene analysis)	Our previous study
DPO-R	GTGATGAGTCTGCCATTGTTTTATATAGCGGG		

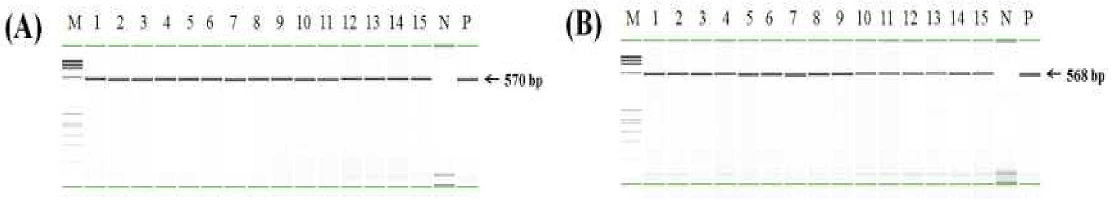
3. Cloning and sequencing of MCP and DPOL genes

For sequence analysis of MCP and DPOL genes, PCR was performed with the M1F/M1R (He et al., 2001) and DPO-F/DPO-R primer sets targeting the full-length sequence of the MCP gene and the partial sequence of the DPOL gene using extracted DNA samples as the template <Table 2>. The PCR conditions for sequence analysis were pre-denaturation at 95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s; followed by an extension period at 72°C for 7 min. The amplified PCR products were cloned to a TA vector (Invitrogen) and the nucleotide sequences were determined using the ABI 3500xl genetic analyzer (Thermo Fisher Scientific, UK).

4. Multiple alignment and phylogenetic analysis of the MCP and DPOL genes

The sequences of both viral genome segments were compared by gene alignment using BioEdit software (ver. 7.0.6 Department of Microbiology, North Carolina State University, Raleigh, NC, USA) to determine the identity of the genotype sequences.

A phylogenetic tree was constructed using ClustalW software (http://www.ebi.ac.uk/clustalW) based on neighbor-joining analysis with MEGA4 (ver. 4.1) (Center for Evolutionary Functional



[Fig. 1]. PCR detection test for megalocytiviruses from diseased fishes of Korean aquatic farms from 2013 to 2017 based on the QIAxcel capillary electrophoresis system. Electrophoresis after PCR using 1-F/1-R (A) and 4-F/4-R primer sets (B). Lane 1, ADC-RSIV2016-2; lane 2, ADC-RSIV2017-18; lane 3, ADC-RSIV2017-22; lane 4, ADC-RSIV2013-24; lane 5, ADC-RSIV2015-24; lane 6, ADC-RSIV2017-27; lane 7, ADC-RSIV2016-29; lane 8, ADC-RSIV2014-35; lane 9, ADC-RSIV2016-38; lane 10, ADC-RSIV2016-41; lane 11, ADC-RSIV2014-44; lane 12, ADC-RSIV2014-46; lane 13, ADC-RSIV2017-49; lane 14, ADC-RSIV2017-68; lane 15, ADC-RSIV2016-56; N, negative control; P, positive control; M, 15 bp ~ 3 kb marker.

(A) Nucleotide

Amino acid	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)
(1) ADC-RSIV2016-2		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(2) ADC-RSIV2017-18	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(3) ADC-RSIV2017-22	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(4) ADC-RSIV2013-24	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(5) ADC-RSIV2015-24	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(6) ADC-RSIV2017-27	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(7) ADC-RSIV2016-29	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(8) ADC-RSIV2016-41	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(9) ADC-RSIV2014-44	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(10) ADC-RSIV2014-46	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(11) ADC-RSIV2017-68	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(12) ADC-RSIV2016-38	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(13) ADC-RSIV2017-49	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8		99.8	99.8	99.9	99.9	99.9	99.9	99.4	97.8	97.9	95.0	95.0	94.5
(14) ADC-RSIV2016-56	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		99.9	100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(15) ADC-RSIV2014-35	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		100.0	100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(16) AP017456	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8		100.0	100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(17) KT804738	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0		100.0	100.0	99.5	97.9	97.9	95.1	95.1	94.6
(18) KC244182	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0		100.0	99.5	97.9	97.9	95.1	95.1	94.6
(19) AY894343	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0		99.5	97.9	97.9	95.1	95.1	94.6
(20) AY532606	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.3	99.6	99.6	99.6	99.6		97.5	97.6	94.9	94.9	94.2
(21) AB104413	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.3	99.6	99.6	99.6	99.6	99.1		99.9	94.2	94.2	94.2
(22) AY779031	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.3	99.6	99.6	99.6	99.6	99.1	100.0		94.3	94.3	94.3
(23) KT781098	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.7	98.7	98.7	98.7	98.2	98.2	98.0		100.0	94.0
(24) AF371960	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.7	98.7	98.7	98.7	98.2	98.2	98.0	100.0		94.0
(25) GQ273492	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	97.8	98.0	98.0	98.0	98.0	97.6	97.6	97.6	98.0	98.0	

(B) Nucleotide

Amino acid	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)
(1) ADC-RSIV2016-2		100.0	100.0	100.0	100.0	100.0	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(2) ADC-RSIV2017-18	100.0		100.0	100.0	100.0	100.0	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(3) ADC-RSIV2017-22	100.0	100.0		100.0	100.0	100.0	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(4) ADC-RSIV2013-24	100.0	100.0	100.0		100.0	100.0	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(5) ADC-RSIV2015-24	100.0	100.0	100.0	100.0		100.0	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(6) ADC-RSIV2017-27	100.0	100.0	100.0	100.0	100.0		100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(7) ADC-RSIV2016-29	100.0	100.0	100.0	100.0	100.0	100.0		99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(8) ADC-RSIV2016-41	100.0	100.0	100.0	100.0	100.0	100.0	100.0		99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(9) ADC-RSIV2014-44	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		99.8	99.8	99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(10) ADC-RSIV2014-46	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		99.8	99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(11) ADC-RSIV2017-68	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0		99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(12) ADC-RSIV2016-38	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5		100.0	99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(13) ADC-RSIV2017-49	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	100.0		99.8	99.8	99.9	99.9	99.9	99.9	99.6	98.0	98.0	94.7	94.7	93.0
(14) ADC-RSIV2016-56	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.0	99.0		99.7	99.8	99.8	99.8	99.8	99.5	97.8	97.8	94.7	94.7	92.9
(15) ADC-RSIV2014-35	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.0	99.0	99.0		99.8	99.8	99.8	99.8	99.5	97.8	97.8	94.8	94.8	93.1
(16) AP017456	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	99.8		100.0	100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(17) KT804738	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	99.8	100.0		100.0	100.0	99.7	98.0	98.0	94.8	94.8	92.9
(18) KC244182	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	99.8	100.0	100.0		100.0	99.7	98.0	98.0	94.8	94.8	92.9
(19) AY894343	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	99.8	100.0	100.0	100.0		99.7	98.0	98.0	94.8	94.8	92.9
(20) AY532606	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	100.0	99.8	100.0	100.0	100.0	100.0		97.8	97.8	95.1	95.1	93.0
(21) AB104413	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.7		100.0	95.0	95.0	93.3
(22) AY779031	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.5	98.7	98.5	98.7	98.7	98.7	98.7	98.7	100.0		95.0	95.0	93.3
(23) KT781098	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.5	96.5	96.7	97.0	96.7	96.7	96.7	96.7	96.7	97.0	97.0		100.0	92.6
(24) AF371960	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.7	96.5	96.5	96.7	97.0	96.7	96.7	96.7	96.7	96.7	97.0	97.0	100.0		92.6
(25) GQ273492	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.7	96.0	96.0	95.7	96.0	95.7	95.7	95.7	95.7	95.7	96.0	96.0	96.5	96.5	

[Fig. 2]. Degree of identity (%) of the nucleotide and deduced amino acid sequence of complete major capsid protein (MCP) (A) and partial DNA polymerase (DPOL) (B) gene in megalocytiviruses of this study to those of other strains.

Genomics, The Biodesign Institute, Tempe, AZ, USA), and nodes were confirmed from bootstrap analysis with 1,000 replicates. The complete sequences of MCP genes and the partial sequences of DPOL genes from megalocytiviruses in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) [Fig. 3] were used to construct the phylogenetic tree for comparison.

Ⅲ. Results

1. Detection of megalocytiviruses from fish tissue samples

We carried out PCR with various primer sets for known fish viruses, including megalocytivirus. However, all 15 samples produced the expected 570 bp and 568 bp amplicons only when running the PCR using both primer sets for megalocytivirus detection, 1-F/1-R and 4-F/4-R, respectively [Fig. 1]. The BlastN analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi) of all sequences from the PCR products obtained using the two primer sets showed more than 99.8% nucleotide sequence identities compared with other known megalocytiviruses (GenBank accession no. AP017456, KT804738, KC244182, and AY894343) belonging to RSIV type-subgroup2.

2. Sequence identity of megalocytivirus MCP and DPOL genes

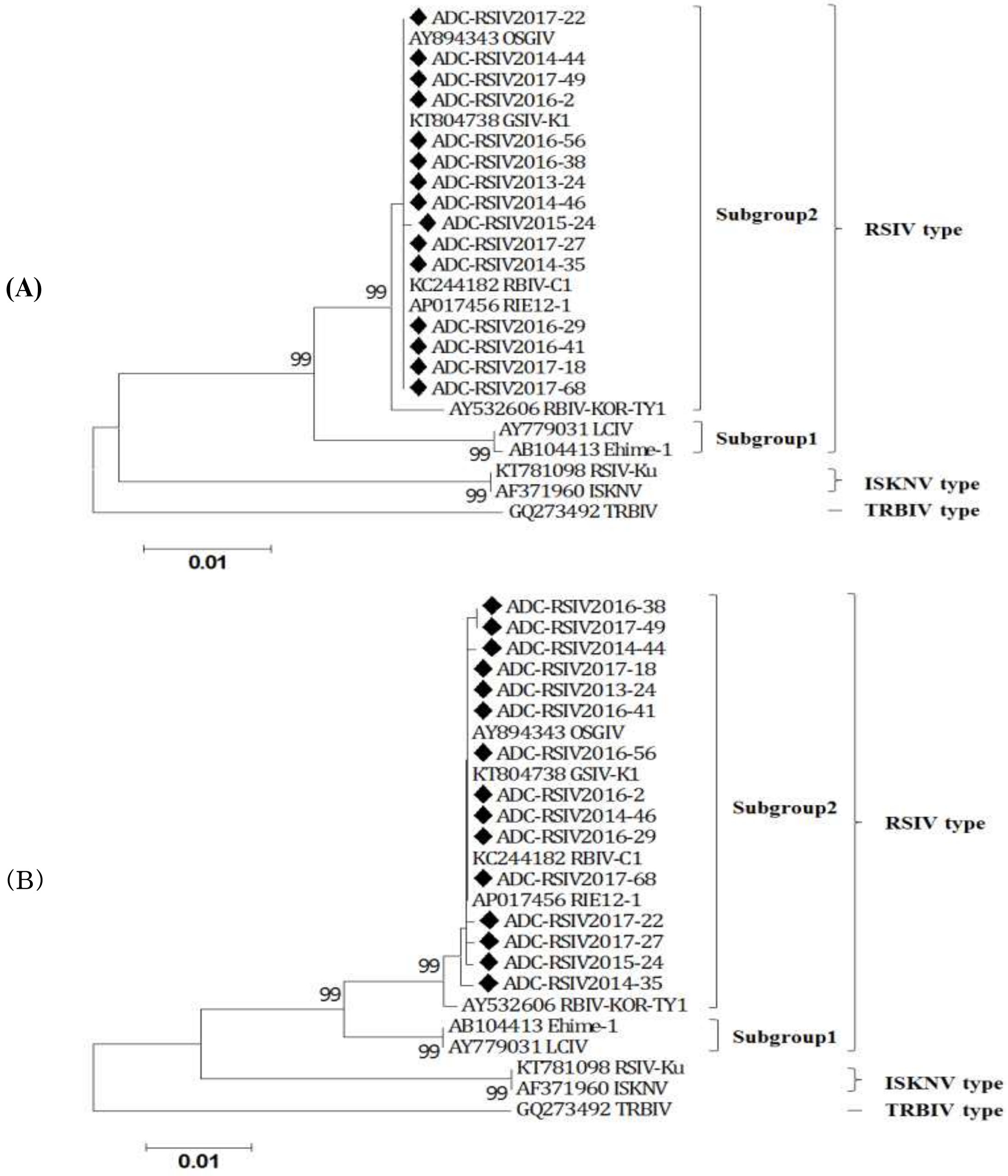
The identities of nucleotide sequence between the obtained virus genes ranged from 99.8 to 100% (amino acid sequence identity: 99.8 to 100%) for the MCP gene and from 99.7 to 100% (amino acid sequence identity: 99.5 to 100%) for the DPOL gene [Fig. 2]. However, comparison of obtained genes with the other virus subgroups ISKNV and TRBIV showed lower sequence identities ranging from 94.5 to 95.1% (amino acid sequence identity: 97.8 to 98.7%) for the MCP gene and from 92.9% to 94.8% (amino acid sequence identity: 95.7 to 97.0%) for the DPOL gene. All of the virus sequences obtained in this study have been uploaded to the NCBI Entrez database (GenBank accession number MK025667 ~ MK025696).

3. Phylogenetic analysis of megalocytiviruses

The phylogenetic tree showed that the nucleotide sequences of both the MCP and DPOL genes comprised three subtypes: RSIV type, ISKNV type, and TRBIV type. The 15 viral strains obtained in this study formed a single cluster of RSIV type-subgroup2 [Fig. 3]. Phylogenetic trees of both the MCP and DPOL genes showed closer clustering of the 15 viral sequences with RIE12-1 and GSIV-K1 isolated in Japan and China than with RBIV-KOR-TY1 isolated in Korea. In addition, the nucleotide sequence divergence from other isolates belonging to RSIV type-subgroup2 was low.

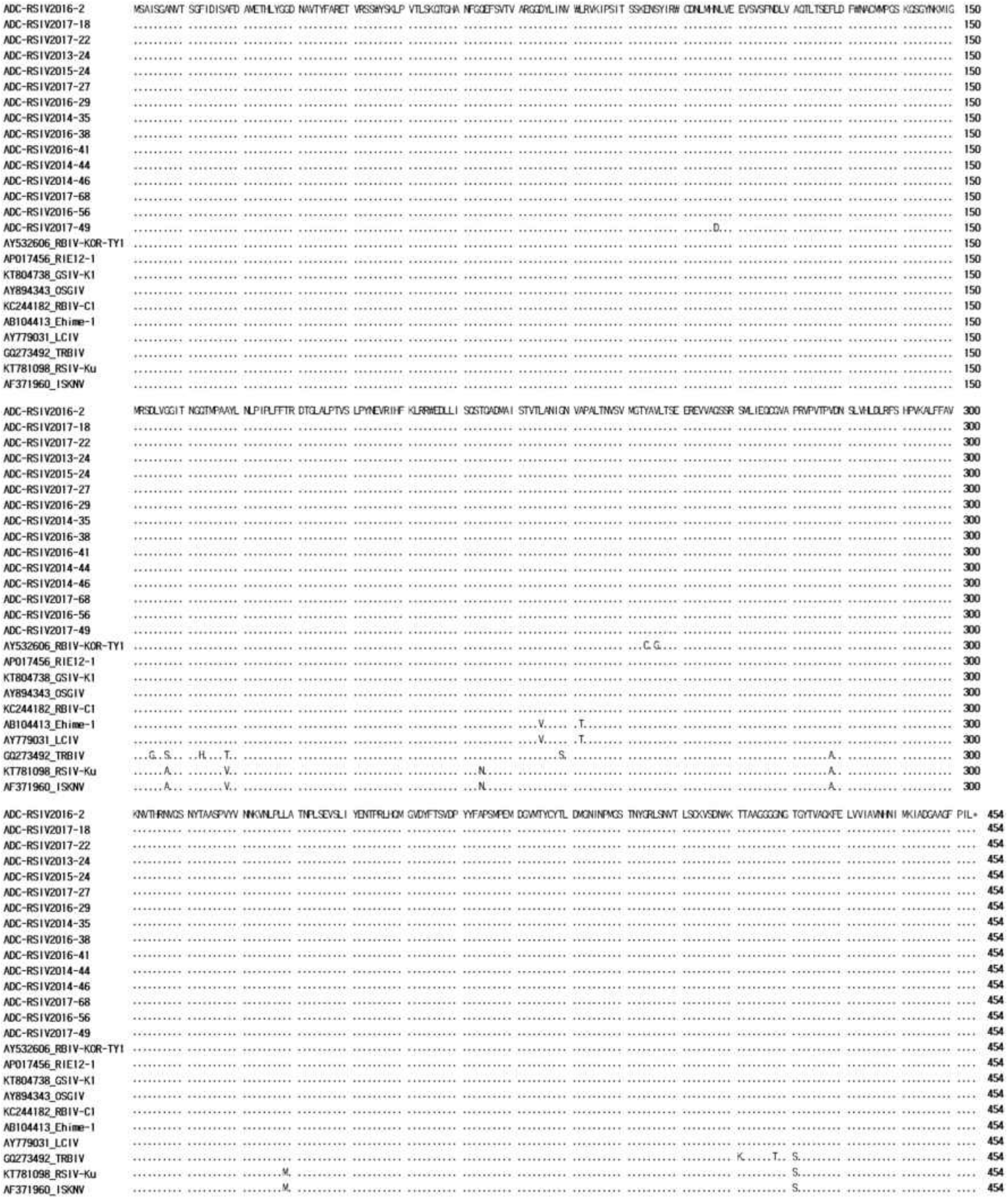
4. Multiple alignments of MCP and DPOL gene sequences in megalocytiviruses

Sequence alignment of the MCP gene among viral strains showed that the majority of the deduced amino acid sequences were identical with only one strain (ADC-RSIV2017-49) showing a single amino acid substitution (N->D at position 107) compared with the others [Fig. 4]. By contrast, for the DPOL gene, three strains contained an amino acid substitution: ADC-RSIV2017-49 (C->R at position 661), ADC-RSIV2016-38 (C->R at position 661), and ADC-RSIV2014-35 (M->T at position 944). Moreover, the DPOL gene of five strains, ADC-RSIV2017-22 (aa 867), ADC-RSIV2017-27 (aa 871), ADC-RSIV2015-24 (aa 804), ADC-RSIV2014-44 (aa 909), and ADC-RSIV2014-35 (aa 945), showed a silent mutation in a single region, in which there was no amino acid substitution despite the nucleotide substitution.



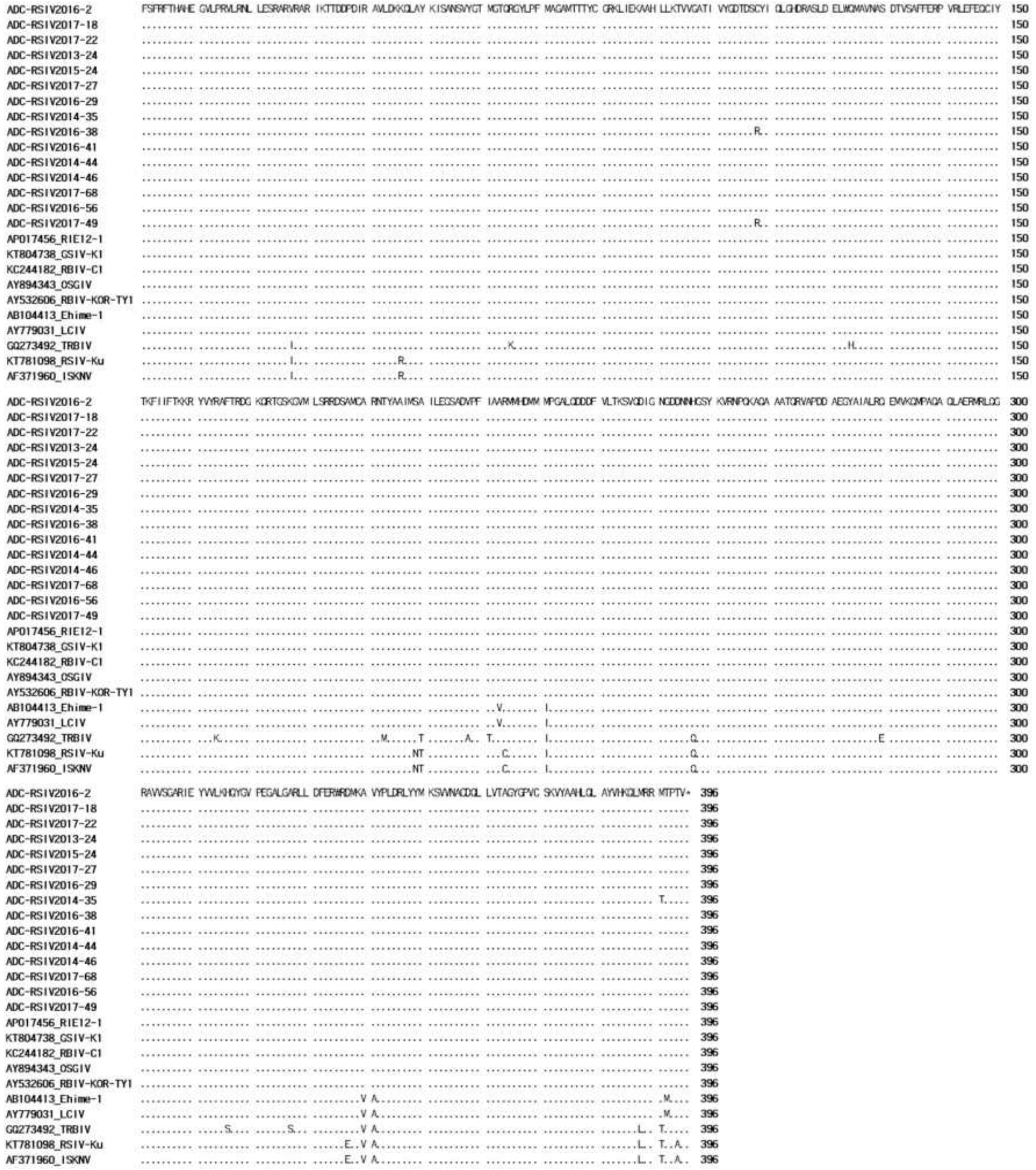
[Fig. 3]. Molecular phylogenetic tree by neighbor-joining analysis constructed based on the complete sequences of megalocytiviruses MCP and DPOL genes. The trees were constituted to compare the phylogenetic relationship of MCP gene (A) and DNA polymerase gene (B) with other megalocytivirus genes from Entrez Database. Bootstrap values were obtained from 1,000 replicates and these values less than 90 were hidden in the tree. The scale bar represents 0.01 nucleotide substitution per site. We marked analyzed sequences of this study (◆).

(A)



(Continued)

(B)



[Fig. 4]. Comparison of the deduced amino acid sequences of complete MCP (A) and partial DPOL (B) genes of megalocytiviruses of this study with known strains, each representing 3 subtypes of Genus *Megalocytivirus*. Amino acid sequences written in alphabet, instead of dots which are represented identical amino acid residues, refer to sequence substitution compared with genes of representative ADC-RSIV2016-2.

Ⅳ. Discussion

RSVID, one of the major pathogenic agents in Korean marine aquaculture, is listed as a notifiable viral disease by the OIE. RSIVD, including RBIVD, is listed as a third-class communicable disease, requiring movement control and disinfection in Korea. In this study, we cloned the MCP and DPOL genes of megalocytiviruses obtained from tissues of infected fish and performed phylogenetic analysis and comparisons of genetic variation to investigate the evolution of megalocytiviruses in Korea from 2013 to 2017. We detected megalocytiviruses in moribund or dead fishes showing typical clinical symptoms of iridoviral disease with PCR using two different primer sets of the OIE Manual of Diagnostic Tests for Aquatic Animals [Fig. 1] : the 1-F/1-R of PCR primer set can amplify partial gene of RSIV and ISKNV type, whereas 4-F/4-R can amplify only the RSIV type. In addition to RSIVD, TRBIVD has also been reported in Korea (Do et al., 2005; Won et al., 2013), and there is no specific detection method for TRBIVD in Manual of Diagnostic Tests for Aquatic Animals of OIE. Therefore, to determine the genetic subtype of megalocytivirus that caused the iridoviral diseases in our sample, we are now developing a duplex PCR method that can detect both the RSIV and TRBIV types so as to be able to quickly prevent spread of the disease through rapid diagnosis.

The MCP and DPOL genes represent structural and non-structural genomic regions of megalocytiviruses, respectively, that are highly conserved, and thus serve as useful targets for evolutionary investigations. Megalocytivirus was previously divided into three subtypes, including RSIV, ISKNV, and TRBIV, based on the MCP gene (Do et al., 2004; Do et al., 2005; Song et al., 2008; Jeong et al., 2006). RSIV type-subgroup2 was shown to be widely distributed in various fish species worldwide, especially in Korea, Japan, China, and other Asian countries (Song et al., 2008; Wang et al., 2009; Zhang et al., 2012; Inouye et al., 1992). Consistently, our phylogenetic analysis demonstrated that megalocytiviruses can be divided into three clusters represented by RSIV, ISKNV, and TRBIV [Fig. 3]. Moreover, even though the MCP and DPOL genes of these viruses that have been present in Korea for the last 5 years clustered more closely with RIE12-1 and OSGIV from Japan and China than with RBIV-KOR-TY1 from Korea [Fig. 3], all of these virus genes still showed high identities (>99.8%) with RBIV-KOR-TY1 [Fig. 2]. This pattern suggests that RSIV type-subgroup2 in Korea have evolved in relation to other RSIV strains existing in the marine environment throughout East Asia.

Because a large number of fishes are transported into domestic aquaculture for commercial and ornamental use, gaining an understanding of the viral host range is important for the control of introducing exotic types from other countries and for disease prevention. Although megalocytiviruses were detected in Korean rockfish as well as rock bream as the main hosts, and were even found over a broad area, including Jeju Island <Table 1>, their gene sequences were still found to be nearly identical. Thus, the clustering pattern of the viral genes of RSIV-subgroup2 is not dependent on the host species, geographical location, or isolation year.

Multiple alignment of the MCP and DPOL nucleotide sequences also demonstrated a lower rate of genetic variation compared with the other strains [Fig. 4]. Our findings support that DNA viruses can evolve slowly to develop genetic variations with distinct amino acid substitutions (Kurita and

Nakajima, 2012; Huang et al., 2011). However, the greater variation of nucleotide sequences implies that evolution of megalocytiviruses is nevertheless underway, and thus mutant strains may emerge, including novel pathogenic types. Therefore, it is necessary to conduct functional analysis of the amino acid substitutions and their potential relation to pathogenicity.

Taken together, the present results based on phylogenetic analysis and genetic comparisons suggest that megalocytivirus isolates from different marine fish in Korea belong to RSIV type-subgroup2 with minimal genetic variation compared with genes of reported strains. This study could provide valuable insight and novel genetic information of recently isolated megalocytiviruses in Korea, laying a foundation for the development of effective vaccines for preventing the spread of megalocytivirus disease in the aquaculture industry.

References

Chinchar G, Essbauer S, He JG, Hyatt A, Miyazaki T, Seligy V and Williams, T(2005). Family Iridoviridae, p.145~162. In Fauquet CM, Mayo MA, Maniloff J, Desselberger E, Ball LA (Eds.), Virus Taxonomy, Eighth report of the International Comittee on Taxonomy of Viruses.Academic Press, San Diego, USA. http://doi.org/10.1007/978-3-540-68618-7_4.

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 and Park JW(2004). Complete genomic DNA sequence of rock bream iridovirus. Virology 325, 351~363. http://doi.org/10.1016/j.virol.2004.05.008.

Do JW, Cha SJ, Kim JS, An EJ, Lee NS, Choi HJ, Lee CH, Park MS, Kim JW, Kim YC and Park JW(2005). Phylogenetic analysis of the major capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea. Dis Aquat Org 64, 193~200. http://doi.org/10.3354/dao064193.

He JG, Wang SP, Zeng K, Huang ZJ and Chan SM(2000). Systemic disease caused by an iridovirus-like agent in cultured mandarin fish, *Siniperca chuatsi* (Basilewsky), in China. J. Fish Dis.. 23, 219~222. http://doi.org/10.1046/j.1365-2761.2000.00213.x.

Huang SM, Tu C, Tseng CH, Huang CC, Chou CC, Kuo HC and Chang SK(2011). Genetic analysis of fish iridoviruses isolated in Taiwan during 2001–2009. Arch Virol 156, 1505~1515. http://doi.org/10.1007/s00705-011-1017-9.

Inouye K, Yamano K, Maeno Y, Nakajima K, Matsuoka M. Wada Y and Sorimachi M.(1992). Iridovirus infection of cultured red sea bream, *Pagrus major*. Fish Pathol 27, 19~27. http://doi.org/10.3147/jsfp.27.19.

Jeong JB, Jun LJ, Yoo MH, Kim MS, Komisar JL and Jeong HD(2003). Characterization of the DNA nucleotide sequences in the genome of red sea bream iridoviruses isolated in Korea. Aquaculture, 220, 119~133. http://doi.org/10.1016/S0044-8486(02)00538-0.

Jeong JB, Jun LJ, Park KH, Kim KH, Chung JK, Komisar JL and Jeong HD(2006). Asymptomatic iridovirus infection in various marine fishes detected by a 2-step PCR method. Aquaculture 255, 30~38. http://doi.org/10.1016/j.aquaculture.2005.12.019.

Jin JW, Kim YK, Hong S, Kim YC, Kwon WJ and Jeong HD(2018). Identification and Characterization of Megalocytivirus Type 3 Infection with Low Mortality in Starry Flounder, *Platichthys stellatus*, in Korea J World Aquacult Soc 49, 229~239. http://doi.org/10.1111/jwas.12439.

Jung MH, Nikapitiya C, Vinay TN, Lee J and Jung SJ(2017a). Rock bream iridovirus (RBIV) replication in rock bream (*Oplegnathus fasciatus*) exposed for different time periods to susceptible water temperatures. Fish Shellfish Immunol 70, 731~735. http://doi.org/10.1016/j.fsi.2017.09.038.

Jung MH, Lee J, Ortega-Villaizan M, Perez L and Jung SJ(2017b). Protective immunity against Megalocytivirus infection in rock bream (*Oplegnathus fasciatus*) following CpG ODN administration. Vaccine 35. 3691~3699. http://doi.org/10.1016/j.vaccine.2017.05.073.

Jung MH, Jung MH, Vinay TN, Nikapitiya C, Kim JO, Lee JH, Lee J and Oh MJ(2015). Effects of water temperature on mortality in Megalocytivirus-infected rock bream *Oplegnathus fasciatus* (Temminck et Schlegel) and development of protective immunity. J Fish Dis 38, 729~737. http://doi.org/10.1016/j.fsi.2017.09.038.

Jung MH, Lee J and Jung SJ(2016). Low pathogenicity of FLIV (flounder iridovirus) and the absence of cross-protection between FLIV and RBIV (rock bream iridovirus). J Fish Dis 39, 1325~1333. http://doi.org/10.1111/jfd.12459.

Jung SJ and Oh MJ(2000). Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. J Fish Dis 23, 223~226. http://doi.org/10.1046/j.1365-2761.2000.00212.x.

Jun LJ, Jeong JB, Kim JH, Nam JH, Shin KW, Kim JK, Kang JC and Jeong HD(2009). Influence of temperature shifts on the onset and development of red sea bream iridoviral disease in rock bream *Oplegnathus fasciatus*. Dis Aquat Org 84, 201~208. http://doi.org/10.1016/j.fsi.2017.09.038.

Kim KI, Hwang SD, Cho MY, Jung SH, Kim YC and Jeong HD(2018). A natural infection by the red sea bream iridovirus type Megalocytivirus in the golden mandarin fish *Siniperca scherzeri*. J Fish Dis 41, 1229~1233. http://doi.org/10.1111/jfd.12815.

Kurita J and Nakajima K(2012). Megalocytiviruses. Viruses, 4, 521~538. http://doi.org/10.3390/v4040521.

Kurita J, Nakajima K, Hirono I and Aoki T(1998). Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). Fish Pathol 33, 17~23. http://doi.org/10.3147/jsfp.33.17.

OIE (World Organisation for Animal Health) (2018) Red Sea bream iridoviral disease. In:Manual of diagnostic tests for aquatic animals. www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_rsbid.htm (accessed 10 Feb 2018).

Rimmer AE, Whittington RJ, Tweedie A and Becker JA(2016). Sus-ceptibility of a number of Australian freshwater fishes to dwarf gour-ami iridovirus (Infectious spleen and kidney necrosis virus). J Fish Dis 40, 293~310. http://doi.org/10.1111/jfd.12510.

Shi CY, Wang YG, Yang SL, Huang J and Wang QY(2004). The first report of an iridovirus-like agent infection in farmed turbot, *Scophthalmus maximus*, in China. Aquaculture 236, 11~25. http://doi.org/10.1016/j.aquaculture.2003.11.007.

Song JY, Kitamura SI, Jung SJ, Miyadai T, Tanaka S, Fukuda Y, Kim, SR and Oh MJ(2008). Genetic variation and geographic distribution of Megalocytiviruses. J Microbiol 46, 29~33. http://doi.org/10.1007/s12275-007-0184-6.

Subramaniam K, Shariff M, Omar AR and Hair-Bejo M(2012). Megalocytivirus infection in fish. Rev Aquaculture 4, 221-233. http://doi.org/10.1111/j.1753-5131.2012.01075.x.

Wang CS, Chao SY, Ku CC, Wen CM and Shih HH(2009). PCR amplification and sequence analysis of the major capsid protein gene of megalocytiviruses isolated in Taiwan. J Fish Dis 32, 543~550. http://doi.org/10.1111/j.1365-2761.2009.01043.x.

Whittington RJ, Becker JA and Dennis MM(2010). Iridovirus infections in finfish – critical review with emphasis on ranaviruses. J Fish Dis 33, 95~122. http://doi.org/10.1111/j.1365-2761.2009.01110.x.

Won KM, Cho MY, Park MA, Jee BY, Myeong JI and Kim JW(2013). The first report of a megalocytivirus infection in farmed starry flounder, *Platichthys stellatus*, in Korea. Fish Aquat Sci 16, 93~99. http://doi.org/10.5657/FAS.2013.0093.

Zhang BC, Zhang M, Sun BG, Fang Y, Xiao ZZ and Sun L(2013). Complete genome sequence and transcription profiles of the rock bream iridovirus RBIV-C1. Dis Aquat Org 104, 203~214. http://doi.org/10.3354/dao02587.

Zhang M, Xiao ZZ, Hu YH and Sun L(2012). Characterization of a megalocytivirus from cultured rock bream, *Oplegnathus fasciatus* (Temminck & Schlege), in China. Aquacult Res 43, 556~564. http://doi.org/10.1111/j.1365-2109.2011.02861.x.

- Received : 04 October, 2018
- Revised : 21 October, 2018
- Accepted : 06 November, 2018