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NOTE

Detection of megalocytivirus from imported tropical ornamental fish, paradise fish *Macropodus opercularis*

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ABSTRACT: Megalocytivirus was detected from paradise fish *Macropodus opercularis* imported from Indonesia. Four of 11 fish (36%) in 2006 and 40 of 117 fish (34%) in 2008 were found to be PCR-positive for megalocytivirus. Phylogenetic analysis based on partial major capsid protein (MCP) gene nucleotide sequences revealed that the sequences detected in paradise fish were classified as Genotype II, which includes freshwater fish isolates from Southeast Asian countries, closely related to infectious spleen and kidney necrosis virus (ISKNV), Murray cod iridovirus (MCIV), and dwarf gourami iridovirus (DGIV-2004). Paradise fish was added as a new host for megalocytivirus based on this study.

KEY WORDS: Megalocytivirus · Paradise fish · *Macropodus opercularis* · Major capsid protein gene · MCP gene

INTRODUCTION

Iridoviruses are large, cytoplasmic DNA viruses with an icosahedral capsid of approximately 200 ± 50 nm in diameter. The family *Iridoviridae* is divided into 5 genera consisting of *Iridovirus, Lymphocystivirus, Chloriridovirus, Ranavirus*, and *Megalocytivirus* (Chinchar et al. 2005). Of these genera, viruses belonging to the genus *Megalocytivirus* have been causing mass mortalities in various marine and freshwater fishes of Asian countries, and much attention has been paid to these viruses because of their economic and ecological impacts on Asian aquaculture (He et al. 2000, Jung & Oh 2000, Sudthongkong et al. 2002a, Kim et al. 2005, Go et al. 2006). Infectious spleen and kidney necrosis virus (ISKNV) and red seabream iridovirus (RSIV) are representative examples of these agents (Inouye et al. 1992, He et al. 2000). The histological characteristics of fish infected with the virus are systemic cell enlargement and necrosis of splenocytes and hematopoietic cells (Inouye et al. 1992, Sudthongkong et al. 2002a, Kim et al. 2005).

Megalocytivirus isolates can be divided into 3 genotypes based on the major capsid protein (MCP) gene sequences (Sudthongkong et al. 2002b, Do et al. 2005, Wang et al. 2007, Song et al. 2008). Genotype I includes isolates from various marine fish species in Japan, Korea, China, and Thailand. Genotype II comprises isolates recovered from freshwater fish species in Southeast Asian countries including China, Indone-

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sia, and Malaysia, and marine fishes caught in the South China Sea. Genotype III is mainly composed of isolates from flatfish species in Korea and China.

In Korea, the first outbreak due to megalocytivirus infection was reported from cultured striped beakperch *Oplegnathus fasciatus*, red seabream *Pagrus major*, and black rockfish *Sebastes schlegeli* in southern coastal areas (Oh et al. 1999, Jung & Oh 2000). Since then, megalocytivirus has been detected not only from turbot *Scophthalmus maximus* in aquaculture (Kim et al. 2005), but also from wild marine fishes from the southern coast of Korea and the East China Sea (Lee et al. 2007). Moreover, recent outbreaks extended the host ranges to imported ornamental fishes (Lyu et al. 2006, Jeong et al. 2008b).

Here we report the detection of megalocytivirus in paradise fish *Macropodus opercularis*, a freshwater ornamental species imported from Indonesia. One hundred percent cumulative mortalities of paradise fish occurred during acclimatization after importation. During the routine examination of these mortalities, megalocytivirus was detected and genetic analysis conducted.

MATERIALS AND METHODS

Experimental fish. In total, 128 paradise fish (body weight 1.2 to 3.3 g) were used for detection of megalocytivirus (Table 1). Fish were imported from Indonesia to Korea in 2006 and 2008, and immediately transported to the laboratory for acclimatization. They were placed into 30 l glass tanks with freshwater and maintained at 23°C. Mortality started on the first day after transportation and continued until 100% cumulative mortality was recorded by Day 3. Freshly dead or moribund fish observed during acclimatization were submitted for examination.

DNA extraction. Internal organs including stomach, intestine, kidney, and spleen were removed from each fish. Each sample was homogenized with tissue culture medium (minimum essential medium, Sigma) in a tube with Precellys 24 (Bertin) at 6000 rpm for 30 s. A 50 µl aliquot of Proteinase K (1 mg ml^{-1}, TaKaRa) was added to 500 µl of the supernatant and incubated for 2 h at 55°C. Total DNA was extracted from the supernatant with phenol-chloroform and precipitated with 0.3 M sodium acetate and ethanol. After centrifugation at 15 000 × *g* for 20 min at 4°C, the pellet was resuspended with 20 µl of diethyl pyrocarbonate (DEPC)-distilled water (Bioneer) and used for PCR template.

PCR. PCR primers mF1 (5′-AGA CCC ACT TGT ACG GCG-3′) - mR1 (5′-CCC ATG TCC AAC GTA TAG C-3′) (primary PCR) and mF2 (5′-CGT GAG ACC GTG CGT AGT-3′) - mR2 (5′-AGG GTG ACG GTC GAT ATG-3′) (nested PCR) were used for amplification of a 1080 and 562 base region of the ISKNV MCP gene, respectively (Wang et al. 2007). PCR amplification was conducted in AccuPower PCR premix (Bioneer) containing 20 pmol of each primer, 40 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 250 µM each dNTP, 1 unit of *Taq* DNA polymerase, and 1 µl of template DNA. The mixture was pre-incubated in a thermal cycler (MyGenie 96 thermal block, Bioneer) at 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C, 1 min extension at 72°C, and 1 min post-extension at 72°C. If the primary PCR was negative, a nested PCR was performed using the same conditions as above. One microliter of the primary PCR product was used as the DNA template for the nested PCR. The amplified PCR products were analyzed in 1.5% agarose gels containing ethidium bromide and visualized under UV light.

Sequence analysis. The PCR products were purified using a gel purification kit (Bioneer). The purified products were cloned with a TOPO-TA cloning kit (Invitrogen) and subjected to nucleotide sequence analysis using an ABI PRISM 3730 XL DNA Analyzer (PE Applied Biosystems). The resulting sequences were assembled with Genetyx Win Ver. 5.1 software, and multiple alignments were constructed using Clustal X (Thompson et al. 1997) to infer genetic relationships among each sequence with the neighbor-

Table 1. *Macropodus opercularis.* Paradise fish samples examined in this study and the results of PCR analysis. Detection rate is expressed as (no. positive samples/no. examined samples). Nested PCR was performed with the primary PCR-negative samples. NT: not tested

Sampling date	Fish body weight (g)	No. of fish	Detection rate of megalocytivirus		
			Primary PCR	Nested PCR	Total positive rate (%)
September 2006	1.9–3.3	11	4/11	NT	4/11 (36)
March 2008	1.3–1.9	27	10/27	7/17	17/27 (63)
June 2008	1.4–2.2	40	1/40	3/39	4/40 (10)
July 2008	1.8–2.5	26	3/26	1/23	4/26 (15)
August 2008	1.2–2.0	24	6/24	9/18	15/24 (62)
Total		128			44/128 (34)

joining algorithm. The final phylogenetic tree was drawn with the MEGA4 program (Tamura et al. 2007). Nucleotide sequences of 31 megalocytiviruses from the public domain were used for comparative analyses.

RESULTS

One hundred percent cumulative mortalities of imported paradise fish occurred during acclimatization. Affected fish lost appetite, showed lethargic swimming on the first day after transportation, and subsequently died in 1 to 2 d. Moribund individuals had no specific external signs but showed discoloration of liver and splenomegaly when postmortem examination was conducted (data not shown).

When PCR was undertaken on 128 fish to detect megalocytivirus, 44 individuals in total were PCR-positive (Table 1). In 2006, 4 of 11 individuals were PCR-positive (36%). In 2008, 17 of 27 individuals (63%) in March, 4 of 40 individuals (10%) in June, 4 of 26 individuals (15%) in July, and 15 of 24 individuals (62%) in August were PCR-positive.

The PCR products (1080 bp) from the one September 2006 and one March 2008 sample were sequenced and named PF06 and PF08, respectively. The nucleotide sequences were registered in GenBank (accession numbers GU168573, GU168574). When the nucleotide sequences of the partial MCP gene (1044 bp, except the primer region) of PF06 and PF08 were compared, the nucleotide sequences showed 99.6% identity with each other (Table 2), and more than 92.5% identity with the sequences of 31 other megalocytiviruses. A phylogenetic tree based on the published MCP gene sequences, including the isolates in this study, revealed that PF06 and PF08 from paradise fish were classified as Genotype II (Fig. 1, Table 3).

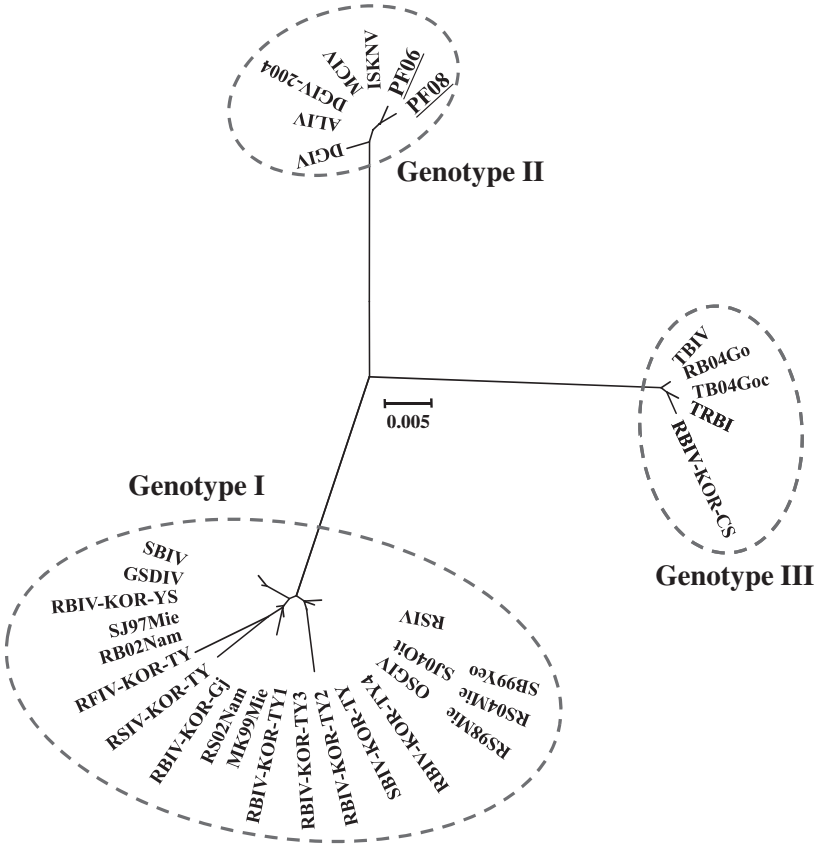

Fig. 1. Molecular phylogenetic tree of the genetic relationship among 33 megalocytiviruses (see Table 3) based on major capsid protein (MCP) gene nucleotide sequences. PF06 and PF08 (underlined) are the 2 new isolates found in this study. Distance marker refers to the expected number of substitutions per site

DISCUSSION

Megalocytivirus was detected from imported paradise fish with a relatively high prevalence of infection (34% in total, Table 1), and 2 isolates

Table 2. Pairwise comparisons (%) of nucleotide sequence identities of the major capsid protein (MCP) gene among 7 megalocytiviruses

Megalocytiviruses	GenBank no.	Genotype	PF06	PF08	RSIV	ISKNV	MCIV	DGIV-2004	TBIV
PF06	GU168573	II	100	99.6	94.7	99.8	99.8	99.8	94.3
PF08	GU168574	II		100	94.7	99.8	99.8	99.8	94.3
RSIV	AY310918	I			100	94.8	94.9	94.9	94.3
ISKNV	AF371960	II				100	100	100	94.4
MCIV	AF371960	II					100	99.9	93.8
DGIV-2004	AY989901	II						100	93.8
TBIV	AB166788	III							100

Table 3. GenBank accession numbers of virus isolates in Fig. 1

Isolate	Accession no.	Source
Genogroup I		
SBIV	AB109372	
GSDIV	AY285746	
RBIV-KOR-YS	AY532610	
RBIV-KOR-GJ	AY532609	
SJ97Mie	Same as RBIV-KOR-GJ	Song et al. (2008)
RB02Nam	Same as RBIV-KOR-GJ	Song et al. (2008)
RS02Nam	Same as RBIV-KOR-GJ	Song et al. (2008)
MK99Mie	Same as RBIV-KOR-GJ	Song et al. (2008)
RSIV-KOR-TY	AY532612	
RFIV- KOR-TY	AY532614	
RBIV-KOR-TY1	AY532606	
RBIV-KOR-TY2	AY533035	
RBIV-KOR-TY3	AY532607	
SBIV-KOR-TY	AY532613	
OSGIV	AY894343	
RS98Mie	Same as OSGIV	Song et al. (2008)
RS04Mie	Same as OSGIV	Song et al. (2008)
SJ04Oit	Same as OSGIV	Song et al. (2008)
SB99Yeo	Same as OSGIV	Song et al. (2008)
RBIV-KOR-TY4	AY532608	
RSIV	AY310918	
Genogroup II		
PF06	GU168573	
PF08	GU168574	
ISKNV	AF371960	
MCIV	AF371960	
DGIV-2004	AY989901	
ALIV	AY285745	
DGIV	AY285744	
Genogroup III		
TBIV	AB166788	
RB04Goc	Same as TBIV	Song et al. (2008)
TB04Goc	Same as TBIV	Song et al. (2008)
TRBI	AY590687	
RBIV-KOR-CS	AY532611	

were named PF06 and PF08. Although megalocytivirus was detected from the imported paradise fish in this study, it was questionable whether this mass mortality of the imported paradise fish was due to this virus because the enlarged splenocytes, typical of megalocytivirus infection, were not detected in histopathological findings (data not shown). Moreover, *Mycobacterium* sp. and reo-like virus were also detected from diseased fish when parasitological, bacteriological, and virological examinations were performed (data not shown). It is not known to what extent these pathogens have contributed to the mortality. The exact cause of this mass mortality is not clear at this point and warrants further investigation.

A radial phylogenetic tree based on the MCP gene sequences identified the 3 major clusters for Genotypes I to III (Fig. 1), as in Song et al. (2008). PF06 and PF08 in the present study were classified into Genotype II, and are most closely related to ISKNV, Murray cod iridovirus (MCIV), and dwarf gourami iridovirus (DGIV-2004; Fig. 1).

In Korea, since Jung & Oh (2000) reported iridovirus-like infection in striped beakperch, there have been many reports on infection of megalocytiviruses belonging to Genotypes I and III (Do et al. 2005, Kim et al. 2005, Song et al. 2008). However, there has been no report on the detection of megalocytiviruses belonging to Genotype II in Korea until Jeong et al. (2008b) reported the detection of megalocytivirus from imported and domestically hatched freshwater ornamental fishes. Ours is the second report of megalocytivirus of Genotype II in Korea, and paradise fish was added as another host for megalocytivirus.

Unlike farmed food fish, the role of ornamental fish has been relatively neglected in terms of translocation of pathogens and the establishment of diseases (Whittington & Chong 2007). This is partly because ornamental fish are sourced from the tropics or the subtropics, which means the hosts and their pathogens are rarely established in the natural environment of countries in temperate zones. However, several examples show that ornamental fish are not an exception for the translocation of pathogens, and ornamental fish pathogens can result in mass mortalities where susceptible host fish species exist. For example, Go & Whittington (2006) mentioned that megalocytivirus from imported dwarf gourami *Colisa lalia* caused mass mortality in Murray cod *Maccullochella peelii peelii* in Australia. Moreover, Jeong et al. (2008a) recently reported that an isolate of the genus *Megalocytivirus* (PGIV-SP), from freshwater ornamental fish, can infect several marine fish species. These observations show that megalocytivirus can cross host species boundaries and establish a new infection if released to the natural environment where susceptible hosts are available. It is likely that the true host range for megalocytivirus is much larger than the current known host range.

Currently there is no evidence that infected imported paradise fish have established breeding populations in Korea. However, attention should be paid to imported aquarium fishes, and good management practices should be implemented to reduce the risk of translocation of pathogens which can cause epizootics in geographically distant regions (Go et al. 2006, Go & Whittington 2006, Jeong et al. 2008a). Aquarium fish, as well as farmed food fish, should be obtained from countries known to be free of disease and should undergo a period of quarantine and health testing to confirm their negative status.

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