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Genetic Variation and Geographic Distribution of Megalocytiviruses

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Viruses belonging to the genus *Megalocytivirus* in the family *Iridoviridae* have caused mass mortalities in marine and freshwater fish in Asian countries. In this study, partial major capsid protein (MCP) gene of seven Japanese and six Korean megalocytiviruses was sequenced and compared with the known megalocytiviruses to evaluate genetic variation and geographic distribution of the viruses. Comparison of MCP gene nucleotide sequences revealed sequence identity of 92.8% or greater among these 48 isolates. A phylogenetic tree clearly revealed three clusters: genotype I including nine Japanese isolates, thirteen Korean isolates, one Chinese isolates, one Thailand isolate and one South China Sea isolate; genotype II including five freshwater fish isolates in Southeast Asian countries and Australia; and the remaining genotype III mainly consisted of flatfish isolate in Korea and China. This suggests that viruses belonging to the genotype I widely distribute among various fish species in many Asian countries. Conversely, the epidemic viruses belonged to genotype II and III are may be still locally spreading and constrained in their prevalence to the limited host fish species, i.e., genotype II viruses mainly distribute in Southeast Asian countries, whereas genotype III viruses distribute in flatfish species in Korea and China.

***Keywords*:** megalocytivirus, iridovirus, genetic variation, geographic distribution

Iridoviruses are large, cytoplasmic DNA viruses with an icosahedral capsid of approximately 200±50 nm in diameter. The iridovirus genome comprises a single linear dsDNA molecule, of which the structure is circularly permuted and terminally redundant (Darai *et al*., 1983, 1985; Schnitzler *et al*., 1987; Chinchar *et al*., 2005). The family *Iridoviridae* is divided into five genera consisting of *Iridovirus, Lymphocystivirus, Chloriridovirus*, *Ranavirus*, and *Megalocytivirus* (Chinchar *et al*., 2005). Of these genera, *Megalocytivirus* was recently identified as a new genus to the family (Chinchar *et al*., 2005), with the infectious spleen and kidney necrosis virus (ISKNV, He *et al*., 2002), isolated from mandarin fish *Siniperca chuatsi* in China, selected as the type species of the genus. Viruses belonging to *Megalocytivirus* have caused mass mortalities in various marine and freshwater fish species, resulting in significant economic losses to the aquaculture industry throughout Asia (Inouye *et al*., 1992; Chou *et al*., 1998; Jung and Oh, 2000; Kim *et al*., 2002; Sudthongkong *et al*., 2002; Shi *et al*., 2004). The distinguishing characteristics of fish affected with the virus are systemic formation of enlarged cells and necrosis of splenocytes and hematopoietic cells (Sudthongkong *et al*., 2002; Chao *et al*., 2004). Kawakami and Nakajima (2002) reported that the disease caused by a megalocytivirus, termed red seabream iridovirus (RSIV), occurred in 31 cultured fish species (included within orders Perciformes, Pleuronectiformes, and Tetraodontiformes) in Japan. In a recent study, we also isolated a new megalocytivirus, turbot iridovirus (TBIV), from diseased turbot *Scophthalmus maximus* in Korea, indicating that megalocytiviruses have a broad host range (Kim *et al.,* 2005; Oh *et al*., 2006). The viral genome of four megalocytiviruses, RSIV (Ehime-1 strain), ISKNV, rock bream iridovirus (RBIV) isolated from rock bream *Oplegnathus fasciatus*, and orange-spotted grouper iridovirus (OSGIV) isolated from orange-spotted grouper *Epinephelus coioides* have been completely sequenced (He *et al*., 2001; Kurita *et al*., 2002; Do *et al*., 2004; Lü *et al*., 2005). Among these four viruses, the high nucleotide and amino acid identities were observed in many open reading flames (ORFs). Recently, megalocytivirus isolates were divided into three groups based on the major capsid protein (MCP) gene, which is one of the most important genes for analysis of genetic relationships among the family *Iridoviridae* (Do *et al*., 2005a, 2005b; Nakajima and Kurita, 2005). However, the geographic distribution of the three groups is unclear. In the present study, therefore, a partial MCP gene of seven Japanese and six Korean megalocytiviruses isolated from various fish species was sequenced to evaluate genetic variation and geographic distribution among the genus *Megalocytivirus*.

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Materials and Methods

Viruses

Thirteen megalocytiviruses were isolated in the present study: RS02Nam, RS98Mie, RS04Mie, RB04Goc, RB02Nam, RB04Jin, SJ97Mie, SJ04Oit, TB04Goc, SB99Yeo, MK99Mie, YT98Oit, and GS04Oit (Table 1). The first two letters of each isolate name, RS, RB, SJ, TB, SB, MK, YT, and GS, indicate the respective host fish: red seabream *Pagrus major*, rock bream *Oplegnathus fasciatus*, striped jack *Pseudocaranx dentex*, turbot *Scophthalmus maximus*, sea bass *Lateolabrax* sp., mackerel *Scomber japonicus*, yellowtail *Seriola quinqueradiata* and gold striped amberjack *Seriola lalandi*. The following numbers indicate the year of isolation, and the letters afterward represent geographic area where the megalocytivirus was collected; Nam, Goc, Jin, and Yeo, denoted cities in Korea, Namhae, Gochang, Jinju, and Yeosu, whereas Mie and Oit were abbreviations of Mie- and Oita-Prefecture in Japan, respectively. The spleen from diseased fish was homogenized in 10 volumes of Hanks' balanced salt solution (HBSS), and was centrifuged at 2,000×g at 4°C. The supernatant containing virus particles was collected and stored at -80°C until used.

Sequencing of major capsid protein (MCP) gene

For PCR amplification of the MCP gene, viral genomic DNA was extracted from the stored virus suspension using a phenol-chloroform solution and subsequently subjected to PCR, as described by Kim *et al*. (2005). PCR amplification was performed with an AccuPower^{TM} PCR premix kit (Bioneer, Korea) according to the manufacturer's instructions. PCR products were analyzed in 1.5% agarose gels containing ethidium bromide and visualized under UV light. After purification with the QIAquick gel extraction kit (QIAGEN, Germany), amplified PCR products were cloned into the pCR 2.1 vector (Invitrogen, USA) to transform *E. coli* (TOP10, Invitrogen, USA) according to the manufacturer's instructions. Nucleotide sequences were performed using an ABI Prism BigDye terminator cycle sequencing FS ready reaction kit with an automated ABI PRISM 310 DNA sequencer.

Phylogenetic analysis

Nucleotide sequences were assembled using the program Genetyx-Win (Ver. 5.1), and the DNA sequences were aligned using CLUSTAL X (Thompson *et al*., 1997) to search for an optimal phylogenetic tree with neighbor joining criteria. The final phylogenetic tree was drawn with the MEGA 3.0 program (Kumar *et al*., 2004).

Results and Discussion

The approximately 1.3 kb PCR product corresponding to the MCP gene was amplified from all thirteen isolates, and nucleotide sequence analysis revealed that all PCR products were 1,299 bases in length and coded 432 amino acid residues (data not shown). Comparison of the MCP gene nucleotide sequences showed that the identities ranged from 92.8 to 100% among the 48 isolates used in the present study (Table 2). From the results, we confirmed that the

Table 1. Megalocytiviruses isolated in this study

Isolate	Origin/Scientific name of the fish	Isolation region	Isolation year
RS02Nam	Red seabream, *Pagrus major*	Korea (Namhae)	2002
RS98Mie	Red seabream, *Pagrus major*	Japan (Mie)	1998
RS04Mie	Red seabream, *Pagrus major*	Japan (Mie)	2004
RB04Goc	Rock bream, *Oplegnathus fasciate*	Korea (Gochang)	2004
RB04Jin	Rock bream, *Oplegnathus fasciate*	Korea (Jinju)	2004
RB02Nam	Rock bream, *Oplegnathus fasciate*	Korea (Namhae)	2002
SJ97Mie	Striped jack, *Pseudocaranx dentex*	Japan (Mie)	1997
SJ04Oit	Striped jack, *Pseudocaranx dentex*	Japan (Oita)	2004
GS04Oit	Gold striped amberjack, *Seriola lalandi*	Japan (Oita)	2004
MK99Mie	Mackereal, *Scomber japonicus*	Japan (Mie)	1999
SB99Yeo	Sea bass, *Lateolabrax* sp.	Korea (Yeosu)	1999
TB04Goc	Turbot, *Scophthalmus maximus*	Korea (Gochang)	2004
YT98Oit	Yellowtail, *Seriola quinqueradiata*	Japan (Oita)	1998

Table 2. Range of nucleotide identities among different genotypes

	Genotype I	Genotype II	Genotype III
Genotype I	≥97.1		
Genotype II	94.1~95.1	≥99.5	
Genotype III	92.8~94.4	93.1~94	≥98.5

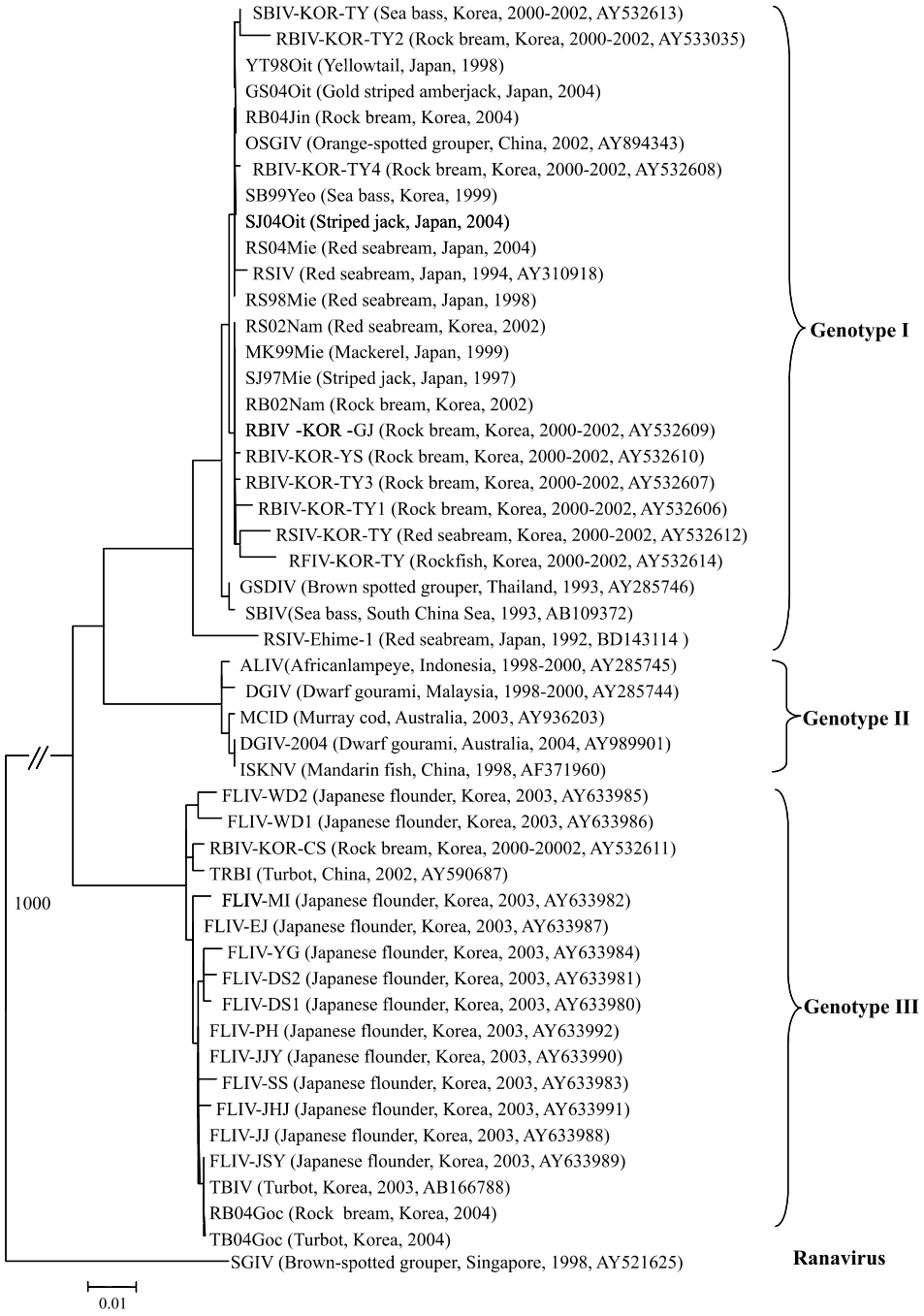

Fig. 1. Molecular phylogenetic tree of the genetic relationship among 48 isolates of megalocytiviruses based on major capsid protein (MCP) gene nucleotide sequence. A ranavirus, SGIV, was used as the out group. Bootstrap values at 1,000 times construction are shown at major nodes in the tree. The scale bar is for a genetic distance marker (number of replacement nucleotides per site).

thirteen Korean and Japanese isolates employed in this study belonged to the genus *Megalocytivirus*. A phylogenetic tree for 48 isolates based on MCP gene nucleotide sequences of 1,299 bases clearly revealed three major clusters as described previously (Fig. 1) (Do *et al*., 2005a; Nakajima and Kurita, 2005). The genotype I included nine Japanese isolates, thirteen Korean isolates, one Chinese isolate, one Thailand isolate, and one South China Sea isolate; the genotype II included five isolates in China, Indonesia, Malaysia, and Australia; and the remaining genotype III included eighteen Korean isolates and one Chinese isolate.

For genotype I, it consisted of 25 viruses isolated from ten different host fish species from various Asian countries, with isolation year ranging from 1992 to 2004. The range of nucleotide sequence identities in genotype I was greater than 97.1% (Table 2). It would appear that these isolates of

megalocytivirus are virologically identical regardless of their host fish species, geographic prevalence and isolation year, but the oldest isolate of the megalocytivirus, RSIV Ehime-1 strain, isolated from red seabream in Japan in 1992 by Nakajima *et al*. (1995), was genetically distant from other viruses belonging to genotype I. Further study is necessary to determine whether the RSIV Ehime-1 strain belongs to genotype I by comparing other genes. Interestingly, we found that all of the Japanese isolates (nine isolates) from five fish species fell into genotype I, even though there were sequence variations of MCP genes among them (>97.9%; data not shown). The geographic distribution of viruses belonging to genotype I seemed to be more widespread to cultured fish in many Asian countries including Japan, Korea, China and Thailand, and the South China Sea (Fig. 2). In addition, we were able to detect by PCR megalocytivirus belonging to the genotype I from wild fish species including blackmouth goosefish *Lophiomus setigerus*, shotted halibut *Eopsetta grigorjewi* and red barracuda *Sphyraena pinguis* caught in the East China Sea near Okinawa Prefecture, Japan (unpublished data). This suggests that the viruses belonging to the genotype I group are widely distributed to cultured and wild fish.

The genotype II group consisted of five viruses isolated from freshwater fish species in China, Indonesia, Malaysia and Australia (Fig. 1); the range of nucleotide sequence identities in the genotype was greater than 99.5% (Table 2), suggesting that the viruses are of a single origin. Nakajima and Kurita (2005) also found that 18 viruses isolated from freshwater and brackish water fish caught in mainly Southeast Asian countries formed one genotype, ISKNV group. From the observations, it is suggested that genotype II viruses are mainly distributed in Southeast Asian countries (Fig. 2). In the case of Australia, Go *et al*. (2006) presumed that MCIV has been introduced to the country via the fish trade from Asia by a molecular epidemiological work. Regarding the host range of the viruses, recently, ISKNV-like viruses were detected from some marine fishes caught in the South China Sea (Wang *et al*., 2007), suggesting that hosts of the viruses belonging to this group consist of both freshwater and marine fishes. There currently are no reports on the detection of genotype II viruses in Japan and Korea, and further surveillance is needed because various live tropical fish species have been transported as ornamental fish from Southeast Asian countries.

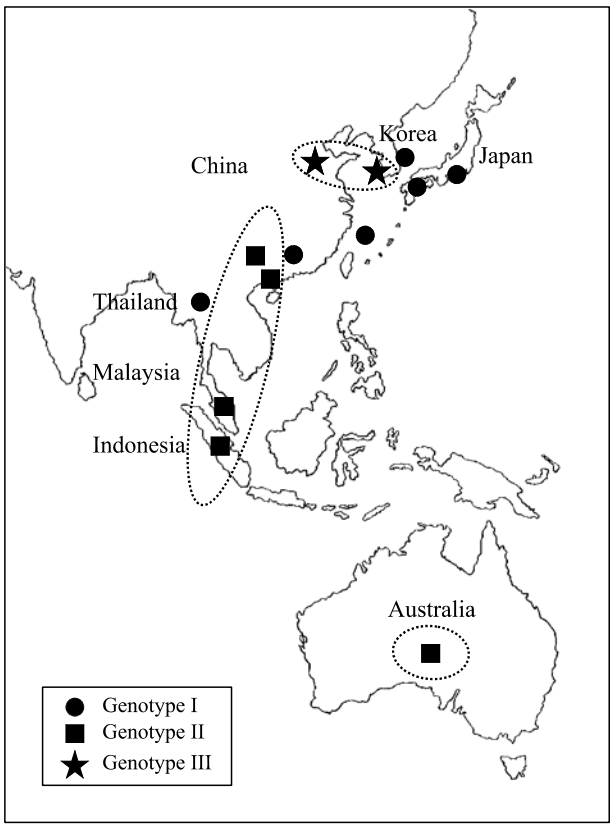

Fig. 2. Geographic location and distribution of three genotypes in the genus *Megalocytivirus*.

On the other hand, genotype III viruses were mainly isolated from flatfish species, including Japanese flounder and turbot in Korea and China (Fig. 1), and the nucleotide sequence identities among viruses in this genotype were greater than 98.5% (Table 2). As in the case of genotype II, genotype III viruses also seemed to share geographic distribution and host fish species, i.e., the incidence of the disease caused by genotype III virus was limited to Korea and China (Fig. 2), and flatfish species were mainly affected by the viruses, although two rock bream isolates (RBIV-KOR-CS and RB04Goc) belonged to genotype III. In Japan, the virus belonging to genotype III has not been isolated thus far, despite Kawakami and Nakajima's (2002) detection of megalocytivirus from Japanese flounder in 1990 to 2000 by indirect immnofluorescence method. Therefore, it is interesting whether these viruses detected from Japanese flounder belong to the genotype III, because live Japanese flounder have been transported from Korea to Japan.

In conclusion, megalocytiviruses isolated from various fish species in many Asian countries were clearly divided into three genotypes (genotype I, II, and III) based on MCP gene sequences. The viruses belonging to genotype I were widely distributed in various Asian countries. Conversely, the viruses belonging to genotype II and III may still be locally spreading and carried in the limited host fish species, i.e., genotype II viruses distributed in China, Indonesia, Malaysia and Australia, whereas genotype III viruses mainly infected flatfish species in Korea and China. The distribution map of the viruses developed in the present study will be useful for tracking of viruses spread internationally via the global transport of live fishes, particularly for genotypes II and III.

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