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Shift of Phylogenic Position in Megalocytiviruses Based on Three Different Genes

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Major capsid protein (MCP), the adenosine triphosphatase (ATPase), and the *Pst*I fragment genes from five Japanese and three Korean megalocytivirus isolates were sequenced and phylogenetically analyzed with known megalocytiviruses. Phylogenetic trees formed three major clusters (M1, M2, and M3 or P1, P2, and P3), and genogroup I was divided into two minor clusters (M1a/M1b and P1a/P1b) using three target genes. Sequence identity was >97% within each cluster, except cluster II of the *Pst*I fragment (>94% of sequence identity). Interestingly, different genotyping patterns were observed for the same isolates depending on the gene analyzed. The JPN-YelTail and JPN-BfTuna isolates located in the minor M1a cluster, based on MCP and ATPase nucleotide sequences, appeared in the minor P1b cluster based on the *Pst*I fragment, suggesting a shift of phylogenic position in megalocytiviruses. Further study will be conducted to compare the viral antigenicity and pathogenicity between the two isolates showing the shift of phylogenic position and the other isolates clustered within genogroup I.

***Keywords*:** Red seabream iridovirus, RSIVD, MCP gene, *Pst*I fragment gene, phylogeny, megalocytivirus

Red seabream iridovirus (RSIV) is the causative agent of RSIV disease (RSIVD), resulting in significant mortality and serious economic losses for more than 30 species of cultured marine fishes in Pacific basin countries (Kawakami and Nakajima, 2002). RSIVD-affected fish become lethargic and exhibit severe anemia, petechiae of the gills, and enlargement of the spleen. The typical histopathological symptoms in an RSIV-affected fish are an enlargement of cells and necrosis of renal and splenic hematopoietic tissues (Inouye *et al*., 1992).

RSIV, a member of the genus *Megalocytivirus* in the family *Iridoviridae,* has an icosahedral capsid measuring 200-240 nm in diameter and a single linear double stranded DNA genome of approximately 111 kb, with a structure that is circularly permuted and terminally redundant as in other members of the family. Megalocytiviruses include infectious spleen and kidney necrosis virus (ISKNV), rock bream (*Oplegnathus fasciatus*) iridovirus (RBIV), turbot (*Scophthalmus maximus*) iridovirus (TBIV), orange-spotted grouper (*Epinephelus coioides*) iridovirus (OGIV), largemouth bass (*Micropterus salmoides*) virus (LMBV), African lamprey (*Aplocheilichthys normani*) iridovirus (ALIV), dwarf gourami (*Colisa lalia*) iridovirus (DGIV), and grouper (*Cromileptes altivelis*) sleepy disease virus (GSDIV) (He *et al*., 2000; Hanson *et al*., 2001; Sudthongkong *et al*., 2002; Do *et al*., 2004; Mahardika *et al*., 2008; Kim *et al*., 2005). Full-length nucleotide sequences of the RSIV, ISKNV, RBIV, and OGIV genomes have been analyzed, identifying 124 putative open reading frames ranging in size from 40-1,208 amino acids (He *et al*., 2001; Do *et al*., 2004; Lü *et al*., 2005). Among them, the major capsid protein (MCP), the DNA polymerase (DPOL), the *Pst*I fragment, and the adenosine triphosphatase (ATPase) genes have been targeted for viral detection and analysis of genetic relationships among megalocytiviruses (Kurita *et al*., 1998; He *et al*., 2001; Sudthongkong *et al.*, 2002; Do *et al.*, 2005a, 2005b; Shinmoto *et al.*, 2009).

Jeong *et al*. (2003) sequenced the K1 region of the RSIV Namhae isolate containing the *Pst*I fragment gene. Two isolates from sea perch imported from China was analyzed by PCR with primer sets targeting the ATPase gene, DPOL gene and ribonucleotide reductase small subunit (RNRS) gene, but not with the primer set targeting the *Pst*I fragment gene (Jeong *et al*., 2004). They suggested that those three genes are relatively highly conserved but the *Pst*I fragment gene has nucleotide variations.

Based on the MCP and ATP genes, megalocytiviruses show >94% nucleotide sequence identities, but only 38-72% nucleotide sequence identities with iridoviruses belonging to the genera, *Iridovirus, Chloriridovirus, Ranavirus*, and *Lymphocystivirus* (Chinchar *et al*., 2005). It was considered that all megalocytiviruses could be members of the same viral species because >93% amino acids sequence identities occur among megalocytiviruses analyzed to date. Moreover, polyclonal anti-RSIV serum shows cross-reactivity with the infected cells of other megalocytiviruses, whereas monoclonal anti-RSIV antibodies react only with RSIV-infected cells (Chinchar *et al*., 2005). Song *et al.* (2008) found that the genotyping of megalocytiviruses correlated with geographic distribution based on the MCP gene nucleotide sequence; the genogroup I (G-I) viruses are widely distributed among various fish species in many Asian countries, genogroup II (G-II) is mainly distributed in Southeast Asian countries, and genogroup III (G-III) is distributed in flatfish species in Korea and China.

Although high genetic similarity is noted in the genus *Megalocytivirus*, the size of the viral particle varies; RSIV,

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Table 1. List of virus isolates used in the present study

Group	Isolate name	Country	Host fish name	Access. No.	Reference	Year	Target gene		
							*Pst*I	MCP	ATPase
Genogroup I	01:JPN-RedSb	Japan	Red seabream (*Pagrus major*)	BD143114	Kurita *et al.* (Patent)	1992	v	v	v
	02:JPN-RedSb	Japan		JN815055-57	This study	2006	v	v	v
	03:JPN-RedSb	Japan		AB461856	Shinmoto *et al.* (2009)	2005		v	
	04:JPN-RedSb	Japan		AY310918	Sudthongkong *et al.* (2002)	1994		v	
	05:JPN-RedSb	Japan		AB263097	Imajoh *et al.* (2007)	2001		v	
				AB263098					v
	06:JPN-RockBr	Japan	Rock bream (*Oplegnathus fasciatus*)	JN815058-60	This study	2008	v	v	v
	07:JPN-YelTail	Japan	Yellowtail (*Seriola quinqueradiata*)	JN815061-63	This study	2008	v	v	v
	08:JPN-YelTail	Japan		AB461855	Shinmoto *et al.* (2009)	2004		v	
	09:JPN-AmJ	Japan	Amberjack (*S. dumerili*)	JN815064-66	This study	2008	v	v	v
	10:JPN-BfTuna	Japan	Bluefin tuna (*Thunnus thynnus*)	JN815067-69	This study	2008	v	v	v
	11:KOR-RedSb	Korea	Red seabream	AY532612	Do *et al.* (2005b)	2000-02		v	
	12:KOR-RedSb	Korea		AB178944	Kitamura *et al.* (unpub.)			v	
	13:KOR-RedSb	Korea		AF487899	Jeong *et al.* (2006)				v
	14:KOR-RockBr	Korea	Rock bream	AY532606	Do *et al.* (2005b)	2000-02	v	v	v
	15:KOR-RockBr	Korea		AY628698	Jeong *et al.* (unpub.)	1999-00	v		
	16:KOR-RockBr	Korea		AY532610	Do *et al.* (2005b)	2000-02		v	
	17:KOR-RockBr	Korea		AY532609	Do *et al.* (2005b)	2000-02		v	
	18:KOR-RockBr	Korea		AY532608	Do *et al.* (2005b)			v	
	19:KOR-RockBr	Korea		AY532607	Do *et al.* (2005b)	2000-02		v	
	20:KOR-RockBr	Korea		AY533035	Do *et al.* (2005b)	2000-02		v	
	21:KOR-RockBr	Korea		AY849393	Kim *et al.* (2007)			v	
	22:KOR-RockBr	Korea		AY849394	Kim *et al.* (2007)			v	
	23:KOR-RockBr	Korea		AB178943	Kitamura *et al.* (unpub.)			v	
	24:KOR-RockBr	Korea		JN815070-72	This study	2004	v	v	v
	25:KOR-RockBr	Korea		JN815073-75	This study	2009	v	v	v
	26:KOR-RockF	Korea	Rockfish (*Sebastes schlegeli*)	AY532614	Do *et al.* (2005b)	2000-02		v	
	27:KOR-JFlound	Korea	Japanese flounder (*Paralichthys olivaceus*)	DQ198145	Kim *et al.* (2007)			v	
	28:KOR-Seabass	Korea	Sea bass (*Lateolabrax japonicus*)	AY532613	Do *et al.* (2005b)	2000-02		v	
	29:KOR-Seabass	Korea	Sea bass (*Lateolabrax* sp.)	AB178942	Kitamura *et al.* (unpub.)			v	
	30:TWN-Barr	Taiwan	Barramundi perch (*Lates calcarifer*)	EU847416	Wang *et al.* (2009)	2005		v	
	31:TWN-Barr	Taiwan		EU847417	Wang *et al.* (2009)	2007		v	
	32:TWN-Barr	Taiwan		EU847418	Wang *et al.* (2009)	2008		v	
	33:TWN-GiSP	Taiwan	Giant seaperch (*Lates calcarifer*)	AY059400	Chao *et al.* (2002)		v		
	34:TWN-GiSP	Taiwan		AF462344	Lai *et al.* (unpub.)				v
	35:TWN-GiSP	Taiwan		EU315313	Wen *et al.* (2008)			v	
	36:TWN-KingGr	Taiwan	King grouper (*Epinephelus lanceolatus*)	EU847414	Wang *et al.* (2009)	2005		v	
	37:TWN-KingGr	Taiwan		EU847415	Wang *et al.* (2009)	2007		v	
	38:TWN-LMBass	Taiwan	Largemouth bass (*Micropterus salmoides*)	AY059401	Chao *et al.* (2002)		v		
	39:TWN-LMBass	Taiwan		AF462345	Lai *et al.* (unpub.)				v
	40:TWN-SilvSb	Taiwan	Silver seabream (*Rhabdosargus sarba*)	EU847419	Wang *et al.* (2009)	2005		v	
	41:TWN-CoPony	Taiwan	Common ponyfish (*Leiognathus equulus*)	EU847420	Wang *et al.* (2009)	2005		v	
	42:CHN-Seabass	China	Sea bass (*Lateolabrax sp.*)	AB109372	Sudthongkong *et al.* (2002)	1993		v	
	43:CHN-Seabass	China		AY310917	Sudthongkong *et al.* (2002)	1993		v	
	44:CHN-Seabass	China		AB043977	Sudthongkong *et al.* (2002)	1993			v
	45:CHN-OrSpGr	China	Orenge spotted grouper (*Epinephelus coioides*)	AY894343	Lai *et al.* (2005)	2002	v	v	v
	46:CHN-LYCro	China	Large yellow croaker (*Pseudosciaena crocea*)	AY165049	Ao *et al.* (2006)	1999-01	v	v	v
	47:THA-BrSpGr	Thailand	Brownspotted grouper (*Epinephelus malabaricus*)	AY285746	Sudthongkong *et al.* (2002)	1993		v	
	48:THA-BrSpGr	Thailand		AB043978	Sudthongkong *et al.* (2002)	1993			v
	49:IDN-AfLamp	Indonesia	African lampeye (*Aplocheilichthys normani*)	AB043979	Sudthongkong *et al.* (2002)	1998-00			v
Genogroup II	50:TWN-Gr	Taiwan	Grouper (*Epinephelus* sp.)	AY059399	Chao *et al.* (2002)		v		
	51:TWN-Gr	Taiwan		AF462343	Lai *et al.* (unpub.)				v
	52:CHN-Manda	China	Mandarin fish (*Siniperca chuatsi*, Basilewsky)	AF371960	He *et al.* (2001)	1998	v	v	v
	53:CHN-RedDrum	China	Red drum (*Sciaenops ocellatus*)	AY158658	Lai *et al.* (unpub.)				v
	54:MYS-DwaGo	Malaysia	Dwarf gourami (*Colisa lalia*)	AY285744	Sudthongkong *et al.* (2002)	1998-00		v	
	55:MYS-DwaGo	Malaysia		AY319288	Sudthongkong *et al.* (2002)	1998-00			v
	56:IDN-AfLamp	Indonesia	African lampeye (*Aplocheilichthys normani*)	AY285745	Sudthongkong *et al.* (2002)	1998-00		v	
	57:AUS-DwaGo	Australia	Dwarf gourami	AY989901	Go *et al.* (2006)	2004		v	
	58:AUS-DwaGo	Australia		AY989902	Go *et al.* (2006)	2004			v
	59:AUS-MurCod	Australia	Murray cod (*Maccullochella peeli peeli*)	AY936204	Go *et al.* (2006)	2003			v
	60:AUS-MurCod	Australia		AY936203	Go *et al.* (2006)	2003		v	

Table 1. Continued

Group	Isolate name	Country	Host fish name	Access. No.	Reference	Year	Target gene		
							*Pst*I	MCP	ATPase
Genogroup III	61:KOR-JFlound	Korea	Japanese flounder	AY633985	Do *et al.* (2005a)	2003		v	
	62:KOR-JFlound	Korea		AY633986	Do *et al.* (2005a)	2003		v	
	63:KOR-JFlound	Korea		AY633982	Do *et al.* (2005a)	2003		v	
	64:KOR-JFlound	Korea		AY633987	Do *et al.* (2005a)	2003		v	
	65:KOR-Jflound	Korea		AY633984	Do *et al.* (2005a)	2003		v	
	66:KOR-JFlound	Korea		AY633981	Do *et al.* (2005a)	2003		v	
	67:KOR-JFlound	Korea		AY633980	Do *et al.* (2005a)	2003		v	
	68:KOR-JFlound	Korea		AY633992	Do *et al.* (2005a)	2003		v	
	69:KOR-JFlound	Korea		AY633990	Do *et al.* (2005a)	2003		v	
	70:KOR-JFlound	Korea		AY633991	Do *et al.* (2005a)	2003		v	
	71:KOR-JFlound	Korea		AY633983	Do *et al.* (2005a)	2003		v	
	72:KOR-JFlound	Korea		AY633988	Do *et al.* (2005a)	2003		v	
	73:KOR-JFlound	Korea		AY633989	Do *et al.* (2005a)	2003		v	
	74:KOR-JFlound	Korea		AY661546	Kim *et al.* (unpub.)			v	
	75:KOR-RockBr	Korea	Rock bream	AY532611	Do *et al.* (2005b)	2000-02		v	
	76:KOR-Turb	Korea	Turbot (*Scophthalmus maximus*)	JN815076-77	This study	2003	v	v	
	77:KOR-Turb	Korea		AB166788	Kitamura *et al.* (unpub.)	2003		v	
	78:CHN-Turb	China	Turbot	AY590687	Shi *et al.* (2004)	2002		v	
	79:CHN-Turb	China		AY608684	Shi *et al.* (2004)	2002			v
	80:CHN-SeaPer	China	Seaperch (*Lateolabrax* sp.)	AY628699	Jeong *et al.* (2006)	2000	v		

200-240 nm (Inouye *et al*., 1992): TBIV, 136-159 nm (Kim *et al.*, 2005): mullet iridovirus-like agent, 100-120 nm, tiger grouper iridovirus-like agent, 210-245 nm (Gibson-Kueh *et al*., 2004), and Singapore grouper iridovirus, 154-176 nm (Qin *et al*., 2003).

Some vaccine experiments have been conducted against RSIV to prevent RSIVD, using formalin-inactivated RSIV culture fluid, recombinant protein, and DNA vaccines (Nakajima *et al*., 1997, 1999, 2002; Park *et al.*, 2005; Caipang *et al.*, 2006; Tamaru *et al.*, 2006; Kim *et al.*, 2008).

The effectiveness of the formalin-inactivated vaccine was determined in several cultured fish under laboratory and field conditions and is available commercially in Japan and Korea. However, some cases of insufficient protection against RSIVD were observed in rock bream from Japan and Korea immunized with the commercial vaccine.

In the present study, the *Pst*I fragment, the MCP gene, and the ATPase gene were phylogenetically analyzed using recent isolates from red seabream, rock bream, yellowtail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), bluefin tuna (*Thunnus thynnus*), and turbot to compare with those of other megalocytiviruses.

Materials and Methods

Viruses

Eight Japanese and Korean RSIV isolates, named 02:JPN-RedSb, 06:JPN-RockBr, 07:JPN-YelTail, 09:JPN-AmJ, 10:JPN-BfTuna, 24:KOR-RockBr, 25:KOR-RockBr, and 76:KOR-Turb, were culture-isolated in 2008 (Table 1). Seventy-three of the deposited megalocytivirus nucleotide sequences in the DNA data bank of Japan (DDBJ) were also used for comparative purposes (Table 1). The two numbers beginning each isolate name are the serial numbers of the viral isolates used in this study, the following three letters indicate the countries of origin, and the last letters indicate the host fish species (Table 1).

RSIV was cultured with grunt fin (GF) cells maintained at 25°C in basal medium eagle (BME) (Sigma, USA), supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 150 IU/ml penicillin. After 2 weeks of culture, the culture fluid was collected, centrifuged at 3,500×g for 10 min to remove cell debris, and stored at -80°C until use.

Polymerase chain reaction (PCR)

The viral genome was extracted from virus culture fluid using a phenol-chloroform extraction method. PCR amplification followed standard methods with three different primer sets. The first primer set was composed of *Pst*1F (5′-CTCAAACACTCTGGCTCATC-3′) or *Pst*I-KF (5′-CTGCAGTTGCCGCTCAAACA-3′) and *Pst*2R (5′-GCGTTAAA GTAGTGAGGGCA-3′), targeting the 848 bp or 860 bp regions of the *Pst*I fragment containing partial RSIV phosphatase and laminin-type epidermal growth factor-like genes (Kurita *et al*., 1998; Jeong *et al.*, 2004). The *Pst*I-KF primer was located 12 bp upstream of the *Pst*1F primer and was used when no PCR product was amplified with the *Pst*1F and 2R set. The second primer set was MCP-F (5′-CAAGT GAGGAGCGTGAGGTTG-3′) and MCP-R (5′-CACAGGATAGGG AAGCCTGC-3′), targeting the 619 bp region of the red seabream iridovirus MCP gene in the GenBank DNA database (accession number AY310918), and the third primer set was ATPase-F (5′-CAAACCAC AGCGCGGCAAGT-3′) and ATPase-R (5′-AGTAGCGCACCATGT CCTCC-3′) targeting the 563 bp region of the ATPase gene (Kurita *et al*., 1998). The extracted viral genome was amplified in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 0.2 μM of each PCR primer, 1.25 U of EX-*Taq* DNA Polymerase (TaKaRa Bio, Japan), 0.2 mM deoxynucleoside triphosphates and 2 mM $MgCl_2$ with a thermal cycler programmed for 1 cycle at 72°C for 10 min and at 95°C for 3 min; 30 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min; followed by a final extension at 72°C for 7 min. Each PCR product was visualized on a 1% agarose-TAE (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) gel and visualized under UV irradiation after ethidium bromide staining.

Sequence analysis

After purification with a PCR purification kit (Stratagene, USA), the amplified viral genome products were subjected to nucleotide

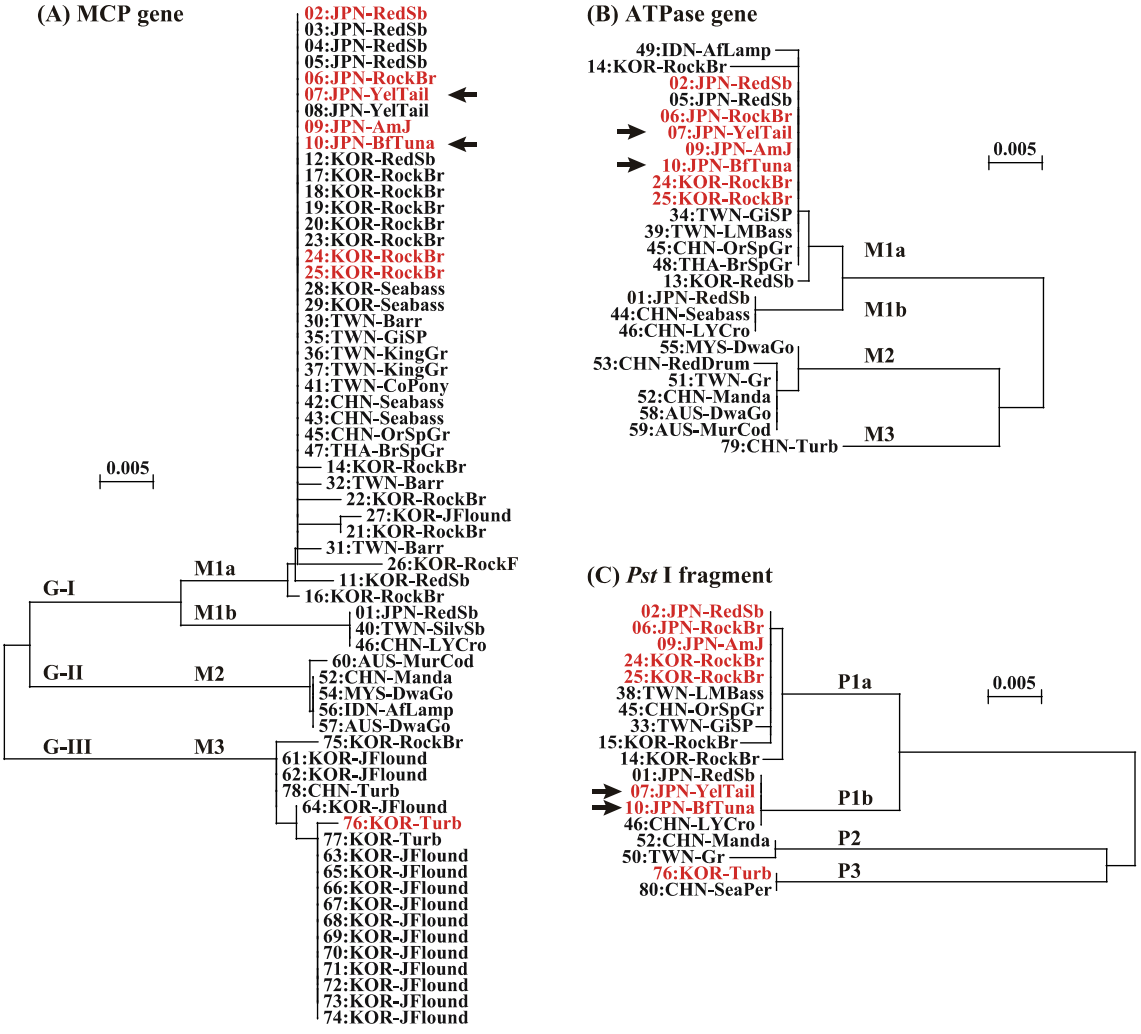

Fig. 1. Phylogenetic trees based on multiple nucleotide sequence alignments of megalocytiviruses. (A) major capsid protein (MCP) gene, (B) ATPase gene, and (C) *Pst*I fragment. Red letters indicate the eight recent isolates analyzed phylogenetically in this study. Arrow indicates two Japanese isolates placed in M1a based on the MCP and ATPase genes, and P1b based on the *Pst*I fragment.

sequence analysis using ABI PRISM Dye Terminator sequencing chemistry (Applied Biosystems, USA) with the PCR primers, according to the manufacturer's instructions. Triplicate PCR products originating from independent amplification reactions were sequenced for each isolate. The resulting sequences were assembled with DNASIS software (Hitachi, Japan) to identify and exclude duplicate sequences from the data set. A multiple alignment of the sequences was constructed using Clustal X based on a single representative of each sequence (Thompson *et al*., 1994, 1997) to infer genetic relationships among sequences with neighbor joining criteria, and a final tree was drawn with NJplot software (Perrière and Gouy, 1996).

Results and Discussion

Amplified products with a size of 620 bp corresponding to the target region were obtained from the MCP gene of all eight RSIV isolates, whereas 560 bp from the ATPase gene were obtained from seven isolates, missing KOR-Turb (#76). PCR products of approximately 850 bp were amplified from the seven isolates (#02, #06, #7, #9, #10, #24, and #25) using the *Pst*1F and 2R primer sets. A PCR product of approximately 860 bp was obtained from the turbot isolate (76: KOR-Turb), using the *Pst*I-KF and 2R primer set, although no product had been obtained using the *Pst*1F and 2R primer set (data not shown). Twenty-three sequences from the eight isolates were deposited in the GenBank nucleotide database (JN815055-JN815076).

Phylogenetic trees based on the determined nucleotide sequences of the *Pst*I fragment, and the MCP and ATPase genes are shown in Fig 1. Sixty-three isolates were divided into three major clusters (M1-M3) in the phylogeny based on the MCP gene. Moreover, the cluster of the 40 M1 isolates was divided into two minor clusters, M1a and M1b (Fig. 1A). The M1a cluster contained 37 isolates from 16 fish species from South Asia to Far East Asia including Japan, Korea, Taiwan, China, Thailand, and Indonesia, and identities of the nucleotide sequence among the M1a isolates were ~99%. The M1b cluster contained three isolates from red seabream in Japan (#01),

large yellow croaker in China (#46), and silver seabream in Taiwan (#40), for which the nucleotide sequences were identical. Approximately 3% nucleotide sequence diversity was observed between the M1a and M1b isolates. The M2 cluster contained five isolates from China, Malaysia, Indonesia, and Australia, and the isolates showed >99% nucleotide sequence identities to each other. The M3 cluster contained 18 isolates from Japanese flounder, rock bream, and turbot from Korea and China, with nucleotide sequence identities >99%. The present results completely agree with the previous genogrouping of megalocytiviruses that correlated with geographic distribution (Song *et al.*, 2008), indicating that M1 corresponded to G-I for isolates widely distributed among various fish species in many Asian countries, M2 corresponded to G-II for isolates distributed mainly in Southeast Asian countries, and M3 corresponded to G-III for isolates limited to Korea and China.

Three major clusters, M1-M3, and two minor clusters, M1a and M2b, were observed in the phylogeny based on the ATPase gene nucleotide sequence. The M1a cluster included 15 isolates from 11 fish species from Japan, Korea, China, Taiwan, and Thailand. The M1b cluster included three isolates from red seabream in Japan (#01) as well as seabass and large yellow croaker from China (#44 and #46). The M2 cluster included six isolates from China, Taiwan, Malaysia, and Australia (#51-#53, #55, #58, and #59), and the M3 cluster contained one isolate from turbot in China (#79). The identities of the nucleotide sequence among isolates of each major or minor cluster were >99%, whereas those between each major cluster were 94-96%.

Three major clusters, P1-P3 and two minor clusters, P1a and P1b, were also observed in the phylogeny based on the *Pst*I fragment nucleotide sequence. The P1a cluster contained 10 isolates from six fish species from Japan, Korea, China, and Taiwan; cluster P1b contained four isolates from red seabream, yellowtail, and bluefin tuna from Japan (#01, 07, and 10, respectively) as well as large yellow croaker from China (#46). The P2 cluster contained two isolates from Mandarin fish from China (#52) and grouper from Taiwan (#50), and the P3 cluster contained two isolates from turbot from Korea (#76) and sea perch from China (#80). Sequence identities between each major cluster were 93-94%.

The 80 isolates used in the present study contained 12 isolates that have not been analyzed previously for the three genes (#1-2, #6-9, #14, #25-26, #45-46, and #52). No difference in the distribution of those isolates in the three major clusters was observed across the analyzed genes. Eleven isolates divided into the major M1 cluster by the MCP gene analysis were also divided into M1 and P1 by the ATPase and *Pst*I fragment gene analyses, respectively. The CHN-Manda isolate (#52) from the M2 cluster by the MCP gene analysis, also appeared in the M2 cluster by the ATPase gene analysis and the P2 cluster by *Pst*I fragment analysis. Similarly, the KOR-Turb isolate (#76) of the M3 cluster by the MCP gene was located in the M3 and P3 clusters by the ATPase gene and *Pst*I fragment gene analysis, respectively. These results indicate that the genotyping of megalocytiviruses correlated with geographic distribution after analyzing the ATPase and MCP gene, as well as the *Pst*I fragment.

Next, we focused on the six Japanese isolates. In the phylogenetic trees based on the MCP and ATPase genes, five of the six Japanese isolates were divided into the minor M1a cluster with complete identity in both the MCP and ATPase sequences, whereas the remaining JPN-RedSb isolate (#01) appeared in the minor M1b cluster. Based on the *Pst*I fragment gene, three Japanese isolates (#02, 06, and 09) were divided into a minor P1a cluster, whereas the other Japanese isolates (#01, 07 and 10) appeared in a minor P1b cluster with complete identity within each subgroup. Even though the JPN-RedSb isolate (#01) was collected 16 years earlier than that of the JPN-YelTail (#07) and JPN-BfTuna (#10) isolates, no difference in *Pst*I fragment nucleotide sequence was observed among these isolates. Therefore, it was concluded that megalocytiviruses could have quite a stable genome with regard to changes in the nucleotide sequence, suggesting that the different M1a and M1b or P1a and P1b minor clusters have been present from the beginning, when RSIV was first found in fish. Interestingly, different genotyping patterns were observed for the same isolates depending on the genes analyzed. The JPN-YelTail (#07) and the JPN-BfTuna isolates (#10) were located in the M1a minor cluster based on MCP and ATPase nucleotide sequences but appeared in the P1b minor cluster based on the *Pst*I fragment sequence. Interestingly, Shinmoto *et al*. (2009) reported that three Japanese isolates with identical MCP nucleotide sequences showed significant differences in viral virulence and antigenicity. It is well known that megalocytiviruses has a single, linear, double-stranded DNA genome. Further study will be conducted to compare the viral antigenicity and pathogenicity between the two isolates (#07 and #10) showing the shift of phylogenic position and the other isolates clustered within genogroup I.

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