Molecular Epidemiology of Koi Herpesvirus

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ABSTRACT—Three regions of koi herpesvirus (KHV) genomic DNA were compared for 34 samples from Japan, six from Indonesia, two from Taiwan, one from the Philippines, 13 from the Netherlands, one from the UK, one from the USA and one from Israel. The analyzed genomic regions included known PCR-detection targets (*SphI*-5, 9/5 and the thymidine kinase gene). The KHVs from Asian countries were very homogeneous, although two variants were noted based on a single nucleotide polymorphism. In contrast, seven variants were found in KHVs from outside of Asia, and although closely related to one another, they were clearly distinct from those from Asian. The results suggest that a clear genetic distinction exists between Asian and European (including each single isolate from the USA and Israel) types of KHV, and that unique types of KHV were independently introduced or emerged in the respective geographic locations.

Key words: koi herpesvirus, KHV, Cyprinid herpesvirus-3, CyHV-3, molecular epidemiology, fish herpesvirus, *Cyprinus carpio*

Koi herpesvirus (KHV), the third cyprinid herpesvirus (CyHV-3) (Waltzek *et al.*, 2005), is highly contagious and can cause disease and mortality in common carp *Cyprinus carpio* and its ornamental variety, koi. The virus was first isolated from fish cultured in Israel and USA, respectively, in 1998 (Hedrick *et al.*, 2000). Subsequently, KHV was also isolated in Germany (Neukirch and Kunz, 2001; Neukirch *et al.*, 2003^{*1}), Israel

(Perelberg *et al.*, 2003; Ronen *et al.*, 2003) and the UK (Way *et al.*, 2001^{*2}). The disease associated with KHV had occurred earlier in Germany in 1997–1998 (Bretzinger *et al.*, 1999) and the presence of this viral

Abstract of the 11th International Conference of the European Association of Fish Pathologists-in Malta.

^{*2} Way, Keith, R.-M. Le Deuff, L. Ecclestone, S. W. Feist, P. F. Dixon, W. H. Wildgoose and R. P. Hedrick (2001): Isolation of a herpesvirus during disease outbreaks in adult koi carp, *Cyprinus carpio*, in the UK. Abstract of the 10th International Conference of the European Association of Fish Pathologists-in Dublin.

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^{*1} Neukirch, M. and D. Steinhagen (2003): Influence of temperature and pH on the infectivity of viruses isolated from koi.

Table 1. List of Japanese KHV samples

Sample No.	Prefecture	Place of sampling	Fish	Farmed or wild	Year	Introduction of common carp from Lake Kasumigaura
1*1	Okayama	river	common carp	wild	2003	no
2*1	Okayama	koi farm	koi	farmed	2003	no
3*1	Okayama	lake	common carp	wild	2003	no
4*1	Okayama	river	koi	wild	2003	no
5*2	Ibaraki	Lake Kasumigaura	common carp	farmed	2003	-
6	Ibaraki	Lake Kitaura	common carp	farmed	2003	yes
7	Miyazaki	fish farm	common carp	farmed	2003	yes
8	Miyazaki	restaurant	common carp	farmed	2003	yes
9	Yamanashi	fishing pond*3	common carp	farmed	2003	yes
10	Mie	fishing pond	common carp	farmed	2003	yes
11	Aomori	fish farm	koi/common carp	farmed	2003	yes
12	Fukuoka	unknown	common carp	unknown	2003	yes
13	Fukuoka	unknown	common carp	unknown	2003	yes
14	Saitama	fishing pond	common carp	farmed	2003	yes
15	Shiga	fish farm	common carp	farmed	2003	yes
16	Osaka	river	common carp	wild	2003	no
17	Niigata	fishing pond	common carp	farmed	2003	unknown
18	Gifu	fishing pond	common carp	unknown	2003	unknown
19	Chiba	fishing pond	common carp	farmed	2003	yes
20	Kagoshima	river	koi	wild	2003	no
21	Kagoshima	river	koi	wild	2003	no
22	Kyoto	river	common carp	wild	2003	unknown
23	Miyazaki	restaurant	common carp	farmed	2003	unknown
24	Tokyo	fishing pond	unknown	farmed	2003	yes
25	Tottori	private household	koi	farmed	2003	no
26	Kyoto	unknown	unknown	wild	2003	unknown
27	Kyoto	unknown	unknown	wild	2003	unknown
28	Kyoto	unknown	unknown	unknown	2003	unknown
29	Shizuoka	fishing pond	common carp	farmed	2003	yes
30	Gifu	unknown	unknown	unknown	2003	unknown
31	Nara	reservoir pond	koi	farmed	2003	no
32	Miyazaki	unknown	common carp	wild	2003	unknown
33	Tottori	private household	koi	unknown	2003	unknown
34	Niigata	unknown	common carp	farmed	2003	yes

*1 : The outbreak of Lake Kasumigaura was preceded by the outbreaks, which became the sources of these samples.

*2 : KHV NRIA-0301 was isolated from this sample.

*3 : A fishing pond is a pond stocked with carp for fee fishing.

infection was confirmed in other European countries and in South Africa (Haenen *et al.*, 2004) since 2001. Recently, KHV was detected by PCR in stored, formalin-fixed tissue from carp sampled in the UK in 1996 (Way *et al.*, 2004*3). In Indonesia, the first outbreak of KHV occurred in fish imported from China through Hong Kong

in 2002 (Yuasa, 2004; Sunarto *et al.*, 2005). In the same year, a mass mortality of carp caused by KHV occurred in Taiwan (Tu *et al.*, 2004).

In Japan, KHV was first detected by PCR from diseased common carp cultured in Lake Kasumigaura in Ibaraki Prefecture in October, 2003 (Sano *et al.*, 2004; Iida and Sano, 2005; Sano *et al.*, 2005). However, the detection of KHV from frozen fish samples from a disease outbreak in Okayama Prefecture revealed that KHV was introduced into Japan as early as May 2003 (Sano *et al.*, 2004). Although there has been speculation, no definitive information is available for the source

*3 Way, Keith, N. D. Beevers, C. L. Joiner, C. B Longshaw, S. St-Hilaire, D. M. Stone, K. L. Denham and P. F. Dixon. (2004): Koi herpesvirus in the UK: Detection in archive samples and spread of the virus to wild carp, Abstract of the 6th International Symposium on Viruses of Lower Vertebrates- in Hakodate.

Table 2. KHV samples collected from various regions of the world and their sequence variations

Country and sample designations	Sample form	Fish	Area	Year	Enlarged 9/5 region		Enlarged Sph I-5 region		Enlarged TK gene region								Genotype/variant ^{*5}	
					212-218		209 586-588		877-885									
					184-187	212-218	209	586-588	94	778	813-814	849/850	945-956	957-958	961-967			
USA (F98-50) ^{*1}	Isolate	Koi	Mid-Atlantic region	1998	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
Israel (R98-21) ^{*1}	Isolate	Koi	Unknown	1998	TTTT	AAAAAA	C	AAC	T	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E6
The Netherlands 1 (625362)	Fixed tissue	Koi	Unknown	2003	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 2 (625967-1)	Fixed tissue	Koi	Unknown	2003	TTTT	AAAAAA	T	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E2
The Netherlands 3 (626095)	Fixed tissue	Koi	Unknown	2003	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 4 (626325)	Fixed tissue	Koi	Unknown	2003	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 5 (626517)	Fixed tissue	Koi	Unknown	2003	—	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E3
The Netherlands 6 (593506)	DNA	Koi	Unknown	2001	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 7 (610783)	DNA	Koi	Unknown	2002	TTTT	AAAAAA	C	AAC	C	G	AT	AA	AA	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E4
The Netherlands 8 (613437)	DNA	Koi	Unknown	2002	TTTT	AAAAAA	C	AAC	C	G	AT	AA	AA	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E4
The Netherlands 9 (04000526)	Fixed tissue	Koi	Unknown	2004	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 10 (04010771)	Fixed tissue	Koi	Unknown	2004	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 11 (04011737)	Fixed tissue	Koi	Unknown	2004	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 12 (04011834)	Fixed tissue	Koi	Unknown	2004	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E1
The Netherlands 13 (04012885)	Fixed tissue	Koi	Unknown	2004	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	—	—	E5
UK (UKD164)	Isolate	Koi ^{*2}	England	2001	TTTT	AAAAAA	C	AAC	C	G	AT	—	—	TTTTTTTT	CTTTAAAAAA	CA	AGATAATT	E7
Japan (NRIA-0301) ^{*3}	Isolate	Common carp	Lake Kasumigaura	2003	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Taiwan 1	DNA	Koi	Ligang in Pingtung County	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Taiwan 2	DNA	Koi	Unknown	2005	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A2
Philippines	DNA	Koi ^{*4}	quarantine station near Manila	2005	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Indonesia 1	Fixed tissue	Common carp	Bengkulu Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Indonesia 2 (2G/3G)	Fixed tissue	Common carp	Riau Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Indonesia 3 (P1/P2)	Fixed tissue	Common carp	West Sumatra Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A2
Indonesia 4 (K1-7G)	Fixed tissue	Common carp	Bengkulu Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Indonesia 5 (K6-2G)	Fixed tissue	Common carp	Bengkulu Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A1
Indonesia 6 (T4-3)	Fixed tissue	Common carp	Lake Tobo, North Sumatra Prov.	2004	TTTT	AAAAAA	C	—	C	A	—	AA	AA	TTTTTT	CTTTAAAAAA	—	AGATAATT	A2

Name of isolate or original sample distinction name in each country is shown by the side of each sample name using parentheses. Numbers located under the names of each analyzed region show nucleotide positions of each variable area and are indicated using nucleotide positions of European genotype variant 1 (E1) as standard. No corresponding sequence in the alignment was indicated using minus symbols. Neighboring variable regions of enlarged TK gene region were divided using vertical dot lines and should be recognized as one variable region.

^{*1}: Hedrick *et al.* (2000) ^{*2}: Imported from Malaysia ^{*3}: In addition to this, other all Japanese KHV's (tissue samples) were classified as Asian genotype variant 1 (A1). ^{*4} Imported from PR China. ^{*5}: The alphabets show genotypes and figures show variants, respectively. For example, E1 means European genotype variant 1 and A2 means Asian genotype variant 2.

of infection which introduced the virus into Japan. In the present study, we compared regions of the KHV genomic DNA from various countries of the world for sequence variations that would provide insights into the potential routes by which KHV has been spread worldwide.

Materials and Methods

KHV samples

Thirty four KHV samples from Japan, six from Indonesia, two from Taiwan, one from the Philippines, 13 from the Netherlands, one from the UK, one from the USA and one from Israel were used for analysis. Japanese samples of KHV were obtained from the tissues (mostly the gills) sent to the National Research Institute of Aquaculture from prefectural governments for the confirmatory diagnosis. In addition, samples from the frozen tissues of dead carp from the outbreaks in Okayama Prefecture (Sano *et al.*, 2004) were also included. The outbreak of Lake Kasumigaura was preceded by these outbreaks.

The information about Japanese and other worldwide samples analyzed in the present study is summarized in Table 1 and Table 2, respectively. The KHV sample from the Philippines was taken from koi transported to a quarantine station near Manila International Airport. The koi were bought during the Aquarama trade show in PR China and imported and confiscated at the airport. KHV samples were collected either as tissues of infected fish, DNA extracts from the tissues, or as isolates of the virus. The samples included common carp and koi, both cultured and wild.

DNA extraction for PCR templates

For tissue samples, DNA was extracted using the Puregene DNA Purification Kit (Gentra Systems, USA) according to the manufacturer protocol, and the extracted DNA pellet was dissolved in 50 μ L of nuclease-free distilled water. For KHV isolates, viruses were inoculated onto KF-1 (Hedrick *et al.*, 2000) or CCB cells (Neukirch and Kunz, 2001) and DNA was then prepared from the supernatant of infected cell cultures using Instagene Matrix (Bio-Rad Laboratories, USA).

Primers and PCR amplification for the three genomic regions of KHV

Primer sequences for amplification of the 610 bp-length 'enlarged *Sph*I-5 region' including the known PCR-detection target of the 290 bp-length '*Sph*I-5' region (Gray *et al.*, 2002) are 5'-CGGAGGACCTGATAACCATCA-3' as the forward primer and 5'-GGTAGC-TGCAGTCGTGCGAC-3' as the reverse primer. Those for the 611 bp-length 'enlarged 9/5 region' including the known PCR-detection target of the 484 bp-length '9/5' region (Gilad *et al.*, 2002) are 5'-TCCAGCGACA-

GGATCTCTCG-3' as the forward primer and 5'-TCTGCGTGACGCTCTCCAAC-3' as the reverse primer. Those for the 1,001 bp-length 'enlarged thymidine kinase (TK) gene region' including 651bp-length TK gene open reading frame and nested 409 bp-length PCR-detection target, 'TK' (Bercovier *et al.*, 2005) are 5'-AACGCG-GGCCAGCTGAACAT-3' as the forward primer and 5'-TGTGTGTATCCCAATAAACG-3' as the reverse primer. Sequence information of upstream and downstream of '9/5' region was kindly provided by Dr. Gilad (pers. comm.). Other sequence information was obtained by genome walking using demi-specific single primer PCR (Morzunov *et al.*, 1995) or LA-PCR between the region and neighboring genomic fragments. The reaction mixture for PCR contained 2 μ L of 10 $\times$ PCR buffer, 1.6 μ L of a 2.5 mM dNTP mixture, 0.5 μ L of each primer (100 pmol/ μ L), 14.7 μ L of sterile distilled water, 0.2 μ L (1 U) of Taq DNA polymerase (Ex Taq Hot Start Version; TaKaRa, Japan), and 0.5 μ L of extracted DNA as template. The thermocycling profile included initial denaturation at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 7 min.

Cloning, sequencing and sequence analyses

The PCR products of expected molecular size by agarose-gel electrophoresis were purified by MinElute PCR Purification Kit (QIAGEN, Germany) and directly sequenced in both orientations with the primers used for amplification or several inner primers (sequences are not shown). When the bands were faint, bands of expected size were collected from the gel and purified by MinElute Gel Extraction Kit (QIAGEN). Then the purified DNAs were ligated into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) and cloned into *Escherichia coli* strain DH5 α . Plasmids from at least five independently derived clones were extracted using QIAprep Spin Miniprep Kit (QIAGEN) and sequenced in both orientations and consensus sequences were determined. All sequencing was performed using ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the ABI PRISM 377 DNA sequencer (Applied Biosystems, USA). Alignments of nucleotide and amino acid sequences were basically made using GENETYX-MAC (GENETYX, Japan).

Results and Discussion

The comparison of the DNA sequences revealed ten variable areas in the three genomic regions of the KHV genome (Table 2). Based on the observed pattern of polymorphism, KHV samples were roughly divided into two groups, designated as Asian and European genotypes with each genotype containing two or seven

E1-3, 6	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
E5	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
E7	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
E4	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
A1	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
A2	376	GAGGTGATGCAGCGTCTGGAGGAATACGACGCCGTGGCCGTCGACGAGGGACAGTTCTTCCCCGACCTCT	445
annealing position of primer TKcomF ↓			
E1-3, 6	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
E5	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
E7	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
E4	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
A1	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
A2	446	ACGAGGGAGTCGTGCAGCTGCTGACCGCGGGCAAGTACGTGATCGTGGCGGCGCTGGACGGGGACTTTAT	515
<div style="display: flex; justify-content: space-between; align-items: center;"> <div> E1-3, 6 E5 E7 E4 A1 A2 </div> <div> 516 516 516 516 516 516 </div> <div> GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG GCAGCAGCCCTTCAAGCAGGTGACGGCGTTGGTGCCCATGGCGGACAAGCTGGACAAGCTGACGGCGGTG </div> <div> 585 585 585 585 585 585 </div> </div>			
E1-3, 6	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
E5	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
E7	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
E4	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
A1	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
A2	586	TGCATGAAGTGCAAGATGCGCGACGCACCCCTTACCCTCAGAACTCTCTCAGGGCAGCGACCTGGTCCAGG	655
<div style="display: flex; justify-content: space-between; align-items: center;"> <div> E1-3, 6 E5 E7 E4 A1 A2 </div> <div> 656 656 656 656 656 656 </div> <div> TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA TTGGAGGCGCCGAGTCTTACCAGGCGGTGTGTCGTCCCTGTCTCAGGGGTTTACGGATGGCCAGTACGA </div> <div> 725 725 725 725 725 725 </div> </div>			
E1-3, 6	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
E5	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
E7	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
E4	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
A1	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
A2	726	GCTGTACGGTCCGCGCCCTCCTCCTCTGCGCATAATCTACTGGGTGCGCCCGTCGTGTACGCCGTCCA	795
stop *			
annealing position of primer TKspecER7C ↓			
E1-3, 6	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAAT--TGTGCGACTTGAAT	863
E5	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAAT--TGTGCGACTTGAAT	863
E7	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAAT--TGTGCGACTTGAAT	863
E4	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAATTAATGTCGACTTGAAT	865
A1	796	CCTCGTTCTTGTAAC--ATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAATAATGTCGACTTGAAT	863
A2	796	CCTCGTTCTTGTAAC--ATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAATAATGTCGACTTGAAT	863
** annealing position of primer TKspecAR3A5A ↓			
E1-3, 6	864	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	933
E5	864	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	933
E7	864	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	932
E4	866	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	935
A1	864	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	931
A2	864	ATGGTTGTACGGGTTTTTTTTTAAACAAAATAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTT	932
** polyA signal stop			
E1-3, 6	934	TGCGTCCAATACTTTAAAAAAACAGGAGATATTAATATAGTTCAAACGTTTATTGGGATACACACA	1001
E5	934	TGCGTCCAATA-----GG-----AAATATAGTTCAAACGTTTATTGGGATACACACA	980
E7	933	TGCGTCCAATACTTTAAAAAAACAGGAGATATTAATATAGTTCAAACGTTTATTGGGATACACACA	1000
E4	936	TGCGTCCAATACTTTAAAAAAACAGGAGATATTAATATAGTTCAAACGTTTATTGGGATACACACA	1003
A1	932	TGCGTCCAATACTTTAAAAAAACAGGAGATATTAATATAGTTCAAACGTTTATTGGGATACACACA	997
A2	933	TGCGTCCAATACTTTAAAAAAACAGGAGATATTAATATAGTTCAAACGTTTATTGGGATACACACA	998

Fig. 1. Multiple alignment of the partial enlarged TK gene sequences. Only partial sequences around the stop codon are shown. Abbreviations of genotype/variant are the same as those in Table 2. Numbers indicate nucleotide positions. Disagreements of nucleotides are indicated by asterisks. Stop codons are denoted by rectangles. The polyadenylation site determined by 3' RACE and annealing positions of newly designed primers to distinguish between Asian and European genotypes of KHV are shown using arrows.

variants, respectively.

Only two variants (A1 and A2) were found in KHV collected from Asian countries based on one base "T" deletion found in serial "T" residues (No. 877–885) in the enlarged TK gene region (Table 2). These Asian variants differed from European KHV variants in six or seven variable areas and formed an Asian genotype, which can be clearly distinguished from the European genotype of KHV mentioned below. In particular, the C-terminal amino acid residues of the TK gene of the Asian genotype varied greatly by frame-shift compared to the European genotype of KHV (Figs. 1, 2). The reference strain of the Japanese KHV, NRIA-0301, was classified as variant A1 and we could not find any sequence differences among all of the KHV samples collected in Japan. This A1 variant was also found in all other Asian countries; Taiwan, Philippines and Indonesia where KHV was obtained for this study. The other variant of the Asian genotype, A2, was not found in Japan or the Philippines but was present in Indonesia and Taiwan. The presence of two variants in Indonesia and Taiwan suggests either that KHV has invaded these areas at least twice, or less likely, the same subtype differentiation occurred independently after a single KHV invasion in these areas. The latter might be possible, because the deletions or insertions in the same serial nucleotide residues are considered to be easy to occur by slippage of the DNA polymerase (Streisinger *et al.*, 1966; Okada *et al.*, 1972; Streisinger and Owen, 1985; Kunkel, 1990; Kroutil *et al.*, 1996; Sia *et al.*, 1997; Strauss, 1999; Gragg *et al.*, 2002; Kühner *et al.*, 2007). In Indonesia, the A2 variant was found only in the west and north of Sumatra Island, whereas all KHV found in east Sumatra was A1. This differential localization of KHV variants in Sumatra Island is in agreement with epidemiological information that the Toba Lake in North Sumatra Province was contaminated with KHV due to the movement of infected juvenile common carp from West Sumatra Province (Sunarto 2004^{*4};

Yuasa, 2007). The homogeneity of Asian KHV suggests that invasion of KHV into these areas occurred more recently and the virus spread rapidly when compared to the case of European KHV mentioned below. In 2002, a KHV outbreak of imported koi occurred in a farm in Guangdong Province of continental China (Liu *et al.*, 2002). Although it was not clear which country the koi were imported from, the reported sequence of 9/5 fragment of this KHV has a characteristic of Asian genotype (the number of serial "A" residues in this region is six and not seven).

In contrast to the Asian genotype of KHV, there were seven variants (E1~E7) in KHV from Europe, the USA, and Israel. However, these non Asian KHV were closely related to each other when they were compared one by one, and thus composed one genetic group that we have denoted European genotype. The results suggest that KHV from the USA, which was isolated in 1998 from the fish returned from a regional koi show (Hedrick *et al.*, 2000), originated from Europe, since the same variant (E1) was also found in Europe. The variant from Israel (E6) was not found in Europe. However, this Israeli KHV, which was isolated in 1998, was closely related to the E1 variant. Hence, it is likely that the Israeli variant also originated from one of the European KHV variants, corresponding to the epidemiological record that KHV was first diagnosed in 1998 after import of koi carp from Europe (Pokorova *et al.*, 2005). The KHV from the UK, which was isolated from koi imported from Malaysia during quarantine, was clearly classified into the European genotype, suggesting the possibility that the koi were infected with KHV in the UK after importation.

When the European variants were compared one by one, differences were found in only one or two genomic regions, although there were six variable regions in total. In contrast, when an Asian variant was compared with any of the European type KHV, differences were found in at least six regions. The presence of several KHV variants in Europe, together with the evidence for the presence of KHV in Europe as early as 1996 (Way *et al.*, 2004^{*3}), suggests that the European genotype of KHV has been present and has diverged for a longer period in Europe than has been observed in Asia. Therefore, it is most likely that Asian KHV were not introduced from Europe and conversely, European KHV did not originate from Asia. Thus, the Asian and European genotypes of KHV are likely to have been independently brought to those regions where they contacted and then caused disease in naive populations of koi and common carp.

Although no intermediate type between the European and Asian genotypes was found in this study,

EURO	1	MAMLELVIGPMFAGKSTESCRRLERLSYSGRRICIAVKHAIDQRYTEESKV	50
ASIA	1	MAMLELVIGPMFAGKSTESCRRLERLSYSGRRICIAVKHAIDQRYTEESKV	50
EURO	51	AMHSGATYPAISAGLYEVMQRLEEDAVAVDEGQFFPDLYEGVVQLLTA	100
ASIA	51	AMHSGATYPAISAGLYEVMQRLEEDAVAVDEGQFFPDLYEGVVQLLTA	100
EURO	101	GKYVIVAALDGFMDQPFQVLTALVPMADKLDKLTAVCMCKMRDAPFTV	150
ASIA	101	GKYVIVAALDGFMDQPFQVLTALVPMADKLDKLTAVCMCKMRDAPFTV	150
EURO	151	RISQGTDLVQVGAESYQAVCRPCLTGFRMAQYELGPPPPPAHNLGA	200
ASIA	151	RISQGTDLVQVGAESYQAVCRPCLTGFRMAQYELGPPPPPAHNLGA	200
EURO	201	PVVSAAAPRSCNISIL	216
ASIA	201	PIVSAAPRSCNIYPVMVCVEPIK	224

Fig. 2. Alignment of the full-length amino acid sequences deduced from the TK gene of European (EURO) and frame-shifted Asian (ASIA) KHV. Numbers mean amino acid positions. Disagreements of amino acid residues are indicated by asterisks.

^{*4} Sunarto, A. (2004): Epidemiology, diagnostic and preventive practices for koi herpesvirus (KHV) in Indonesia. Abstract of

Tokyo University of Marine Science and Technology workshop 'KHV infection –Present Status and Future Prospects for Prevention– in Tokyo.

the insertion of double "A" residues in the enlarged TK gene region of the E4 variant may indicate some relation to the Asian genotype, which has the same insertion. This double "A" insertion located between the polyadenylation signal and polyadenylation site is indispensable to create the new stop codon for the frame-shifted TK gene of the Asian genotype (Fig. 1). This suggests that a possible prototype of KHV had this insertion, which is retained in the E4 variant similar to the Asian genotype while other European variants have lost these residues.

The samples from Europe in this study represent a limited geographic range and additional isolates need to be analyzed. If an intermediate type between the European and Asian genotypes of KHV is found, it would provide a strong indication of the potential origins of the virus. Recently, full genome sequences of two European variants (USA and Israel) of KHV and one Asian variant of KHV were reported (Aoki *et al.*, 2007). A detailed comparison of the genome sequence would present further epidemiological information on KHV.

From the results of the present study, the Asian and European genotypes of KHV can be easily distinguished from each other by typing the frame-shifted region of the TK gene. This will be useful in future epidemiological surveys particularly when the European genotypes of KHV are introduced to Asian countries or vice versa. We propose an easy PCR method to distinguish the two genotypes using a combination of a common forward primer TKcomF (5'-tgcagcgtctggaggaatac-3'), European-type specific reverse primer TKspecER7C (5'-cacaccatcacaggataCatatgt-3'), and Asian-type specific reverse

primer TKspecAR3A5A (5'-cacaccatcacaggatagaAgAta-3') (Fig. 1) under the conditions of a high annealing temperature of 68°C and increased cycles (39 times) (Fig. 3). Each reverse primer was designed from the frame-shifted region of the TK gene and the nucleotides denoted by capital letters in primer sequences indicate intentionally introduced miss match nucleotides to decrease cross reaction. Primer-specific PCR products of 448 or 450 bp are obtained by this method when sufficient KHV DNA is present. The sequence data of each fragment has been deposited in the DDBJ/EMBL/GenBank Data Libraries under accession no. AB375379–AB375391.

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Fig. 3. Agarose gel electrophoresis of PCR products amplified from Asian and European type KHV DNA using the 3 different primer sets. Products were separated on a 1% agarose gel in TBE buffer. Template DNA was prepared from the supernatant of cell culture infected with E1 KHV (Lane 1), the supernatant of cell culture infected with A1 KHV (Lane 2), the koi tissue affected with E1 KHV (Lane 3), the koi tissue affected with A1 KHV (Lane 4) and the non-KHV infected common carp tissue as the negative control (Lane 5). Lane M is the Hyper Ladder II molecular weight marker (BIOLINE, UK). The thermocycling profile was the same as that of 9/5 PCR method by Gilad *et al.* (2002). Each genotype-specific primer set reacted only with the corresponding genotype of KHV, whereas the 9/5 primer set reacted both genotypes of KHV.

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