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Analysis of koi herpesvirus latency in wild common carp and ornamental koi in Oregon, USA

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Koi herpesvirus (KHV) infection is associated with high mortalities in both common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*) worldwide. Although acute infection has been reported in both domestic and wild common carp, the status of KHV latent infection is largely unknown in wild common carp. To investigate whether KHV latency is present in wild common carp, the distribution of KHV latent infection was investigated in two geographically distinct populations of wild common carp in Oregon, as well as in koi from an Oregon-based commercial supplier. Latent KHV infection was demonstrated in white blood cells from each of these populations. Although KHV isolated from acute infections has two distinct genetic groups, Asian and European, KHV detected in wild carp has not been genetically characterized. DNA sequences from ORF 25 to 26 that are unique between Asian and European were investigated in this study. KHV from captive koi and some wild common carp were found to have ORF-25–26 sequences similar to KHV-J (Asian), while the majority of KHV DNA detected in wild common carp has similarity to KHV-U/-I (European). In addition, DNA sequences from IL-10, and TNFR were sequenced and compared with no differences found, which suggests immune suppressor genes of KHV are conserved between KHV in wild common carp and koi, and is consistent with KHV-U, -I, -J.

1. Introduction

Koi herpesvirus (KHV) is highly contagious and pathogenic to both koi and common carp. The first formal descriptions of the disease were from Israel and the USA in 1998 (Calle et al., 1999; Hedrick et al., 1999) and from Germany in 1997–1998 (Bretzinger et al., 1999). The latter group designated the virus as carp nephritis and gill necrosis virus (CNGV) and recently confirmed that CNGV is the same as KHV (Hutoran et al., 2005). The virus has been reported in many countries of Europe, Asia, and North America since 2000. The clinical signs of an active KHV infection include red and white mottling of the gills, gill hemorrhage, sunken eyes, pale patches or blisters on the skin, and external hemorrhages (Gilad et al., 2002). The virus can be found in the kidney, gill, spleen, fin, intestine, and brain (Gilad et al., 2004). More recently, latent KHV has been detected in circulating white blood cells from koi exposure to KHV 10–15 years prior to testing (Eide et al., 2011a,b). In other experimental studies, 82% of fish died within 15 days when they were exposed to the virus at a water temperature of 22 °C (Perelberg et al., 2005).

KHV is formally known as Cyprinid herpesvirus 3 (CyHV-3) and has been proposed to be a member of *Alloherpesviridae* (Waltzek et al., 2005). Members of *Alloherpesviridae* also include CyHV-1 (carp pox herpesvirus) and CyHV-2 (goldfish hematopoietic necrosis virus). Herpes viruses are known to be well adapted viruses in their natural host and can be found in many different species. KHV may have existed in wild common carp before emerging as a highly pathogenic virus. KHV latency sites have been investigated in koi recovered from KHV infection, and no consistent detection of KHV DNA has been found in tissues, such as liver, kidney, or spleen (Eide et al., 2011b; Gilad et al., 2004). However, KHV DNA can be detected consistently in white blood cells of koi that have recovered from a clinical KHV infection (Eide et al., 2010, 2011b), which suggests that white blood cells are the preferred latency site for KHV. To investigate KHV latency in wild carp populations, fish from a flood pond in the Willamette Valley of Oregon and from the Barnyard Springs in the USFWS-Malheur National Wildlife Refuge in Oregon were examined for the presence of KHV genome. In addition, KHV latent infection was investigated in ornamental koi from a fish retailer in Oregon.

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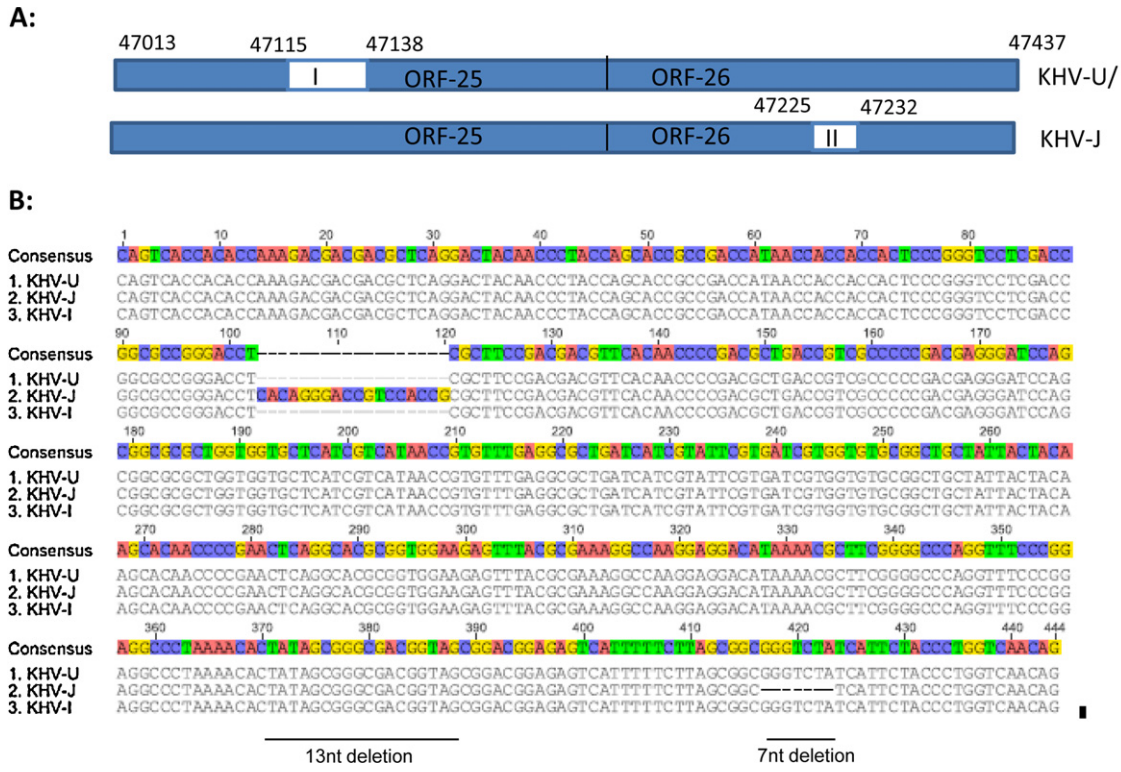

Fig. 1. DNA sequence variations within ORF-25–ORF-26 of KHV-I, KHV-J and KHV-U. (A) Schematic of ORF-25–26 with 13 nt deletion in ORF-25 of KHV-U, -I and 7 nt deletion in ORF-26 of KHV-J. Open box I: 13 nt deletion. Open box II: 7 nt deletion. (B) DNA sequence alignment flanking 13 nt deletion in KHV-U and KHV-I, 7 nt deletion in KHV-J.

There are two genetically distinct KHV groups, European and Asian (Avarre et al., 2011). KHV-I (Israel) and KHV-U (United States) are members of the European group. KHV-U is considered to have originated from KHV-I because of genetic similarity between the two isolates. KHV-J (Japan) belongs to the Asian group, and is genetically different from KHV-U and KHV-I (Aoki et al., 2007; Avarre et al., 2011). The size of the KHV genome is 295, 271; 295, 146, and 295, 138 bp for J, U, and I, respectively (Aoki et al., 2007). Although most genes of the three isolates are similar, ORF-25 and ORF-26 of KHV-J are different from those of KHV-U and KHV-I. As shown in Fig. 1, there is a 13 nt deletion in ORF-25 of both KHV-I and KHV-U that is not observed in ORF-25 of KHV-J. Additionally, KHV-J has a 7 nt deletion in ORF-26 that is not observed in either KHV-I or KHV-U. To investigate whether KHV DNA of wild common carp or ornamental koi is similar to Asian or European KHV, DNA sequences of KHV ORF-25 and ORF-26 from wild carp and koi were investigated and compared in this report.

2. Materials and methods

2.1. Source of wild common carp and sampling

Five wild common carp were obtained from a pond formed from back-water after a flood in Linn County, Oregon, in late spring of 2010. The carp were designated as C1–C5, with ages estimated at 2–5 years old based on morphometric measurements (length and weight). Approximately 2–3 ml of whole blood were collected from the caudal tail vein of each fish and stored in heparin-coated tubes in accordance with National Institutes of Health guidelines and the Oregon State University Animal Care and Use Committee (IACUC). An additional 24 adult wild common carp were collected from Barnyard Springs located in USFWS-Malheur National Wildlife Refuge in Princeton, Oregon in two groups: the first group of 10, designated as A1–A10, was collected in late winter of 2010, the second group of 14, designated as B1–B14, was collected in early spring of 2011. Approximately 1–2 ml of whole blood were collected from each fish and stored at 4 °C in EDTA coated tubes prior to shipping.

2.2. Source of ornamental koi and DNA sampling

Eight 1–2-year old koi ranging from 7 to 10 cm in length, designated as K1–K8, were obtained from a fish supplier in Corvallis, Oregon in the summer of 2010. Because the fish were still very small, DNA was extracted from the entire fish. All koi were euthanized by MS222 overdose at 500 ppm in accordance with National Institutes of Health guidelines and the Oregon State University Animal Care and Use Committee (IACUC). The koi were homogenized in 2 ml 1X lysis buffer, and 0.5 ml of tissue homogenates were digested with 100 μg proteinase K at 55 °C overnight. Genomic DNA was extracted from the tissue lysates with a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

2.3. Separation of peripheral white blood cells (WBC) and total DNA extraction from WBCs and plasma

Whole blood from wild common carp was centrifuged at 650 × *g* at 4 °C for 10 min, the buffy coat was collected and exposed to 3–4 volumes of red blood cell lysis buffer (Tris–NH_4Cl). The white blood cells (WBCs) were washed twice in sterile DMEM by centrifugation at 650 × *g* at 4 °C for 10 min. WBCs were subjected to total DNA extraction using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA).

2.4. Primers and probes

Selection of primers for KHV sequence amplification was based on KHV genome sequence data available through Genbank

Table 1
Primer pairs used to detect KHV DNA in wild common carps and koi.

Name	Gene	Primer sequences (5′–3′)
	DNA polymerase	
KHVDF242		TGTGCGCCAACTCTCACTAC
KHVDR242		GCCCTTGGTGTAGAGGTTCA
KHVNF242		CACGTCCAGAGGGTTCATCT
KHVNR242		AGTCCCTCTGCCAGCATCT
	Major capsid protein	
KHVC-382F		TCTCACCCAGTACACCACCA
KHVC-382R		GTTCATGGCGCCAAAGTAGT
KHVc210F		AGGAGCTGCTGTACCTGGTC
KHVc210R		CTTTCGCGATGTTGTACACG
	ORF25–26	
F525		GGTGGTGCTCATCGTCATAA
R525		TGGTGATGAACTTGGTGGTG
F368		AGGCGCTGATCATCGTATTC
R368		GCAGATGGTACGTGATGCTG
	ORF134 (IL-10)	
F631		AATGTTTGCGCTTGGTTTTC
R631		ACTCCAACCATGTTCCTTGC
F507		TTCTCGACGGATTGGAAGAC
R507		GCCGAAAGATAGTTGCGTGT
	IL-10A	
F507		TTCTCGACGGATTGGAAGAC
R507		GCCGAAAGATAGTTGCGTGT
F301		TCTTTTGCGACCAGGAACTT
R301		CAGCCGCCTATCCAGATAAA
	IL-10B	
F233		GTTGAGGCGAGTTCGTTCAT
R233		ACTCCAACCATGTTCCTTGC
F156		GCAAGGAACATGGTTGGAGT
R156		ATTCCTACTACCCCGCGAAT
	TNFR-N	
F595		ACGCAACAACTCAACAGCAG
R595		GCTCTTGTAGGTGCCCACTG
F474		AGCAGCAAAGAACCCAAAGA
R474		CCGGCTGGTGAGAGGTACT
	TNFR	
F393		ACCTACAAGAGCAACGCACA
R393		GCAGATGGTGTCCTTGTTGA
F265		CCATCATGAGTGTCCCAAGA
R265		CAGGCCAGACTTGGAGATGT
	TNFR-C	
F684		CAGTGGGCACCTACAAGAGC
R684		TCTTGCTCTCGAGTCCTGCT
F437		GACCCAGACCACGGCTACTA
R437		CGCCTAGTGGCCAAAAATAG

(NC_009127). The primers, used for amplification of DNA polymerase, major capsid protein, ORF 25–26, DNA probes specific for ORF-25–26, ORF134 (IL-10), and ORF4 (TNFR) are listed in Table 1 TNFR-N and TNFR-C are primers specific for DNA sequence near the N-terminal and C-terminal, respectively.

2.5. PCR amplification

PCR amplification was performed as follows: a 25 μl solution consisting of 12.5 μl amplification buffer (2X Platinum® PCR Supermix, Invitrogen, Carlsbad, CA, USA), 0.4 μM each primer, and 5 μl of total DNA (0.5–1 μg/μl). The mixture was subjected to 94 °C for 2 min, and 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, followed by a 5 min elongation reaction at 72 °C after the final cycle. A nested-PCR was performed using nested set primers (Table 1). A 3 μl aliquot of the original PCR product was included as a template in the second (nested) amplification.

2.6. Southern blot

PCR products (10 μl of 25 μl total PCR reaction) were electrophoresed through a 1.5% agarose gel, transferred to a nylon membrane (Jin et al., 2000), and then UV cross-linked to the membrane. The DNA products of 10 μl PCR were probed with a digoxigenin-labeled DNA probe. The probe was generated with nested PCR primers that are specific for the target genes. To make digoxigenin-labeled PCR products, digoxigenin-labeled deoxynucleoside triphosphates (Roche Diagnostics, Indianapolis, IN, USA) were added to the PCR mixtures according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). The membrane was incubated in prehybridization buffer (Roche Diagnostics, Indianapolis, IN, USA) at 68 °C, and then hybridized with the Dig digoxigenin-labeled DNA probes specific for gene coding for DNA polymerase at 68 °C. After incubation with the probe, membranes were washed with 0.1% sodium dodecyl sulfate and 10% 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before incubation with an anti-digoxigenin antibody conjugated with peroxidase. The membrane was developed by incubation with a chemiluminescent peroxidase substrate (Roche Diagnostics, Indianapolis, IN, USA). The blots were exposed to film at room temperature for about 30 min. The molecular masses of the resulting bands were estimated by using a 1-kb DNA ladder (Invitrogen, Carlsbad, CA, USA).

2.7. DNA sequencing and analysis

The nested-PCR reaction products were cleaned with a ChargeSwitch PCR Clean-Up kit (Invitrogen, Carlsbad, CA, USA) and sequenced by the Center for Genomic Research and Bioinformatics at Oregon State University. The nucleotide sequences were analyzed with Geneious software.

3. Results

3.1. Detection of KHV DNA in wild common carp from a flood pond in Linn County, Oregon

To determine whether KHV latent infection is present in wild common carp, total DNA extracted from WBC from all five wild common carp was subjected to PCR using primers, KHVDF-242 and KHVDR-242, specific for KHV DNA polymerase gene. To confirm amplified products were indeed KHV DNA sequences, the amplicons were hybridized by a DNA probe specific for the DNA sequence between the two primers' amplification regions as described previously (Eide et al., 2011a). As shown in Fig. 2, KHV DNA was detected in all five carp. To confirm that KHV DNA was detected by PCR and Southern blot, a nested-PCR was used to amplify the conserved major capsid protein using primers KHVC-382F and KHVC-382R in the first PCR reaction, then KHVc210F and KHVc210R in the second PCR reactions as described previously (Eide et al., 2011b). A PCR product at the expected size was amplified from 4 of 5 WBC total DNA samples isolated from the 5 wild common carp. The amplified product was sequenced and was found to be 100% identical to that of KHV-U, -I and -J, respectively (Fig. 2C).

3.2. Detection of DNA in wild common carp from USFWS-Malheur National Wildlife Refuge

To investigate whether KHV latent infection is present in wild common carp in Oregon, blood from two groups (first $n = 10$, the second $n = 14$) of wild common carp was collected in the winter of 2010 and spring of 2011 from Barnyard Springs located in USFWS-Malheur National Wildlife Refuge, Oregon. DNA from the white blood cells was extracted and examined by nested PCR using primers specific for ORF-25–26: F525and R525 in the first PCR reaction, F368 and R368 in the second PCR reaction. As shown in Fig. 3, expected PCR products at 368 bp were detected in 4 of 10 carp from group 1 (A1–A10) and 13 of 14 carp from group 2 (B1–B14). A6 was excluded in the analysis due to the poor quality

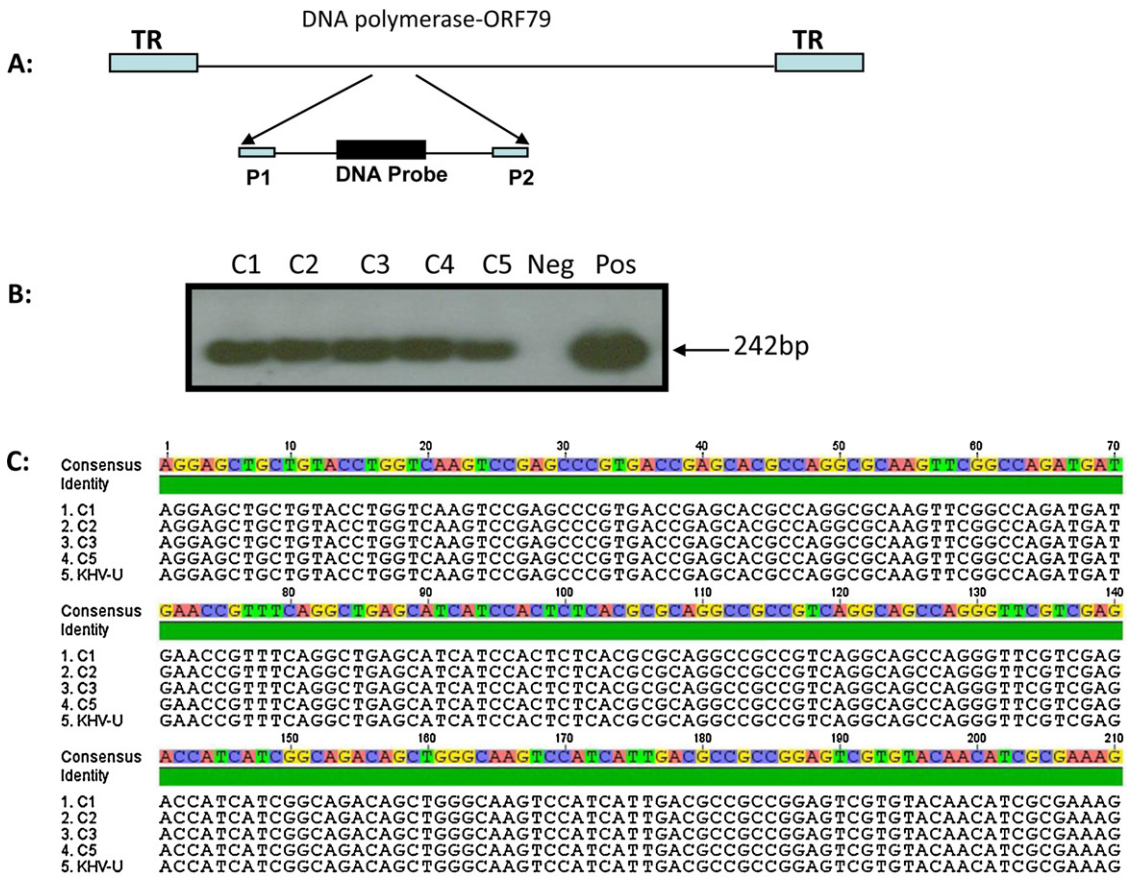

Fig. 2. KHV DNA analysis of total DNA of white blood cells from wild common carp. (A) Schematic of the KHV genome and location of the Southern blot DNA probe within the DNA polymerase gene. TR indicated the viral terminal direct repeats. P1 and P2 indicate the KHV specific primers KHVDF242 and KHVDR242. DNA probe is 158 bp PCR product labeled with digoxigenin (filled box) using primers KHVDNF242 and KHVNDR242. (B) Southern blot analysis of PCR products amplified from white blood cell from C1 to C5. Pos: KHV-U DNA; neg: total DNA of KF-1 cells. (C) DNA sequence alignments of nested PCR products amplified with primers specific for major capsid genes: KHVC-382F and KHVC-382R were used in the first reaction, KHVc210F and KHVc210R in second reaction. C1, C2, C3 and C5: nested PCR products amplified from WBC total DNA from wild common carp. KHV-U: KHV isolate of the United States.

of the blood. Although the amplification of KHV DNA with F525 and R525 in the first reaction was very weak, amplification with nested primers F368 and R368 was visible and specific (Fig. 3). No sign of illness was observed in the wild carp when they were captured at Barnyard Springs. These data suggest that KHV latent infection is present in wild common carp residing in the USFWS-Malheur National Wildlife Refuge.

3.3. Detection of DNA in ornamental koi from a fish supplier in Oregon

Since KHV latent infection was found in wild common carp, it suggests that KHV latent infection could be present in ornamental koi. To determine whether KHV latent infection is present in ornamental koi, 8 fish at 1–2 years old were obtained from an ornamental fish supplier in Oregon. Nested PCR specific for ORF-25–26 was performed as above. As shown in Fig. 4, KHV DNA was detected in 4 of 8 koi total DNA. Since detection using whole fish total DNA is not as sensitive as detection from WBCs, not all fish showed positive results. Koi obtained from the supplier all appeared healthy without any signs of illness. In addition, no KHV associated disease was reported from the supplier where these koi were acquired. Although only 4 koi were found to be positive with KHV infection, these data suggest that KHV latent infection is present and detectable in ornamental koi.

3.4. Analysis of KHV-DNA in wild common carp and ornamental koi

No major KHV outbreaks have been reported from either Barnyard Springs located in USFWS-Malheur National Wildlife Refuge in Oregon or the fish supplier used as a source for ornamental koi. The data suggest that these fish are either infected with a KHV-variant virus that is less pathogenic or they are well adapted to the carp. To determine whether the KHV DNA from wild common carp and ornamental koi is similar to Asian or European KHV, selected nested PCR products amplified with primers specific for ORF-25–26 were sequenced directly. KHV DNA from the 2 ornamental koi is similar to KHV-J (Table 2). As shown in Fig. 5, two KHV DNA (A1and B5) from wild common carp are similar to KHV-J (Asian) which have a 7 nt deletion in the ORF26, while the rest have no deletion in ORF-26 which is similar to KHV-U/I (European). This suggests that KHV of wild common carp has genetic features of both Asian and European groups. To determine whether KHV DNA of wild common carp has 13 nt deletions as described for KHV-U/-I (European), nested-PCR products amplified with primers specific for the region flanking the 13 nt deletion were sequenced. As shown in Table 2, all KHV DNA of wild common carp have the 13 nt deletions as the KHV-U/-I (European), whereas the KHV DNA from the ornamental koi are similar to KHV-J (Asian). These results suggest that the KHV variant of the wild common carp is unique in genetic characteristics and has similarity to both Asian and European isolates of KHV.

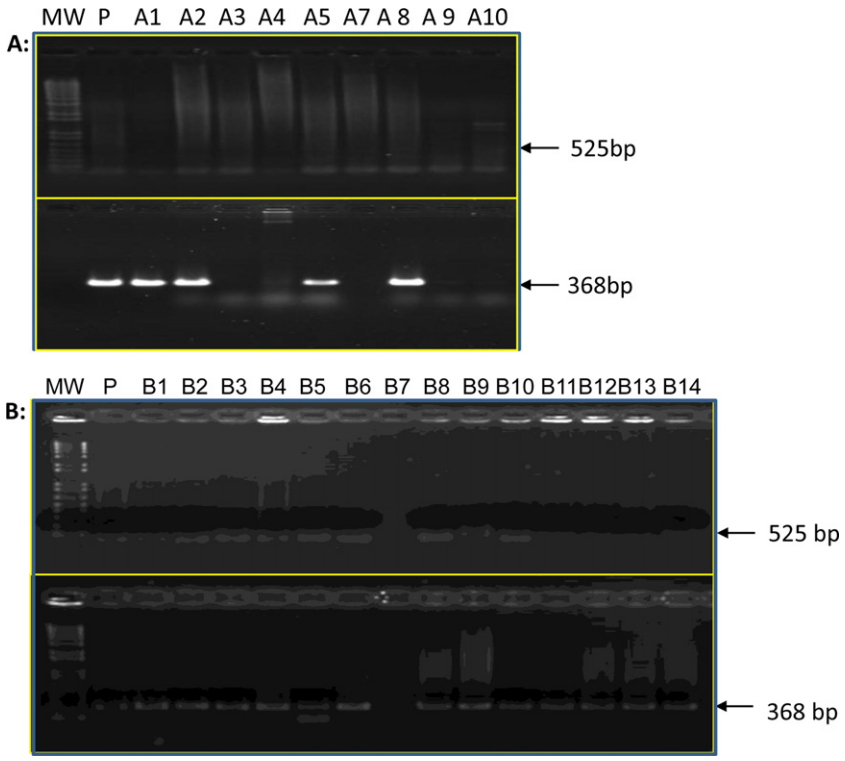

Fig. 3. Detection of KHV DNA in wild common carp by nested PCR. (A) PCR products amplified from total DNA of white blood cell from A1 to A5 and A7 to A10 wild common carp obtained in the winter of 2010. (B) PCR products amplified from total DNA of white blood cells from B1 to B14 wild common carp obtained in the spring of 2011. P: KHV-U DNA. Top panel are PCR products amplified with primers F525 and R525, bottom panel are PCR products amplified with primers F368 and R368.

Table 2
Identification of deletion locus within ORF25 and ORF26.

KHV DNA		ORF25	ORF26
Reference strains	KHV-I	−13 nt	+7 nt
	KHV-U	−13 nt	+7 nt
	KHV-J	**+13 nt**	**−7 nt**
Pet koi	K4	**+13 nt**	**−7 nt**
	K5	**+13 nt**	**−7 nt**
WCC	A1	−13 nt	**−7 nt**
	A2	−13 nt	+7 nt
	B4	−13 nt	+7 nt
	B5	−13 nt	**−7 nt**
	B6	−13 nt	+7 nt
	B8	−13 nt	+7 nt

Bold: deletion locus unique in KHV-J.
Underline: deletion locus unique in WCC.

3.5. Analysis of immune modulating genes

To determine if the KHV variant from wild common carp has variation in immune modulating genes TNFR and IL-10, PCR primers were designed to amplify the coding region of both TNFR and IL-10 of KHV (Table 1). The entire TNFR and IL-10 coding regions were amplified by two or three sets of nested PCR. Nested PCR products were then sequenced and compared to KHV-U and KHV-J. No difference was found in the DNA sequence coding for TNFR (Fig. 6) or IL-6. Therefore, both immune suppressor genes of KHV are conserved between wild common carp KHV and previously characterized KHV isolates from clinical disease outbreaks, such as KHV-U, -I, -J.

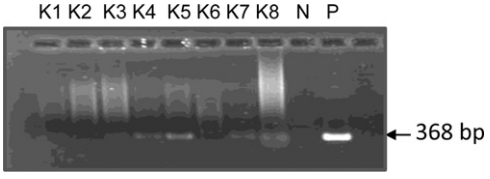

Fig. 4. Detection of KHV DNA in ornamental koi from a fish supplier in Oregon by nested PCR. PCR products amplified from total DNA of entire fish, K1–K8, using nested primers F525 and R525 in first reaction, and then F368 and R368 in the second PCR reaction. P, KHV-U DNA. N: total DNA of KF-1 cells.

4. Discussion

KHV is a recently identified herpesvirus pathogenic to koi (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio carpio*). Initially, KHV was identified in 1998 as the cause of mass mortality among juvenile and adult koi and among common carp cultured in Israel, the United States, and Germany (Bretzinger et al., 1999; Hedrick et al., 1999). Since then, KHV infection has been recognized all over the world. The origin of this virus has been suggested to come from active and often unregulated movements of large numbers of fish, which resulted in rapid spread of the virus presumably from these origins (Aoki et al., 2007). This suggestion is supported by the high degree of similarity between KHV-U and KHV-I isolates. In fact, the first cases of the disease in the eastern United States (strain U) occurred in 1998 following a koi show in New York that involved fish from Israel (Hedrick et al., 1999). The origin of KHV among common carp in Japan is less defined (Sano et al., 2004). The complete genomes of KHV-U, I, and J are 99% identical, with variations that can divide them into two genetic groups: Asian and European. These two genetic groups can also be separated by short tandem repeats, called VNTR (variable number of tandem repeats) or variations within TK gene (Avarre et al., 2011; Kurita et al., 2009). When VNTR was used to genotype 38 samples collected in Indonesia, France, and the Netherlands, it grouped those samples into two main genetic groups: CyHV-3-U/-I and CyHV-3-J. Similarly, when variations of TK genes were used as a marker to

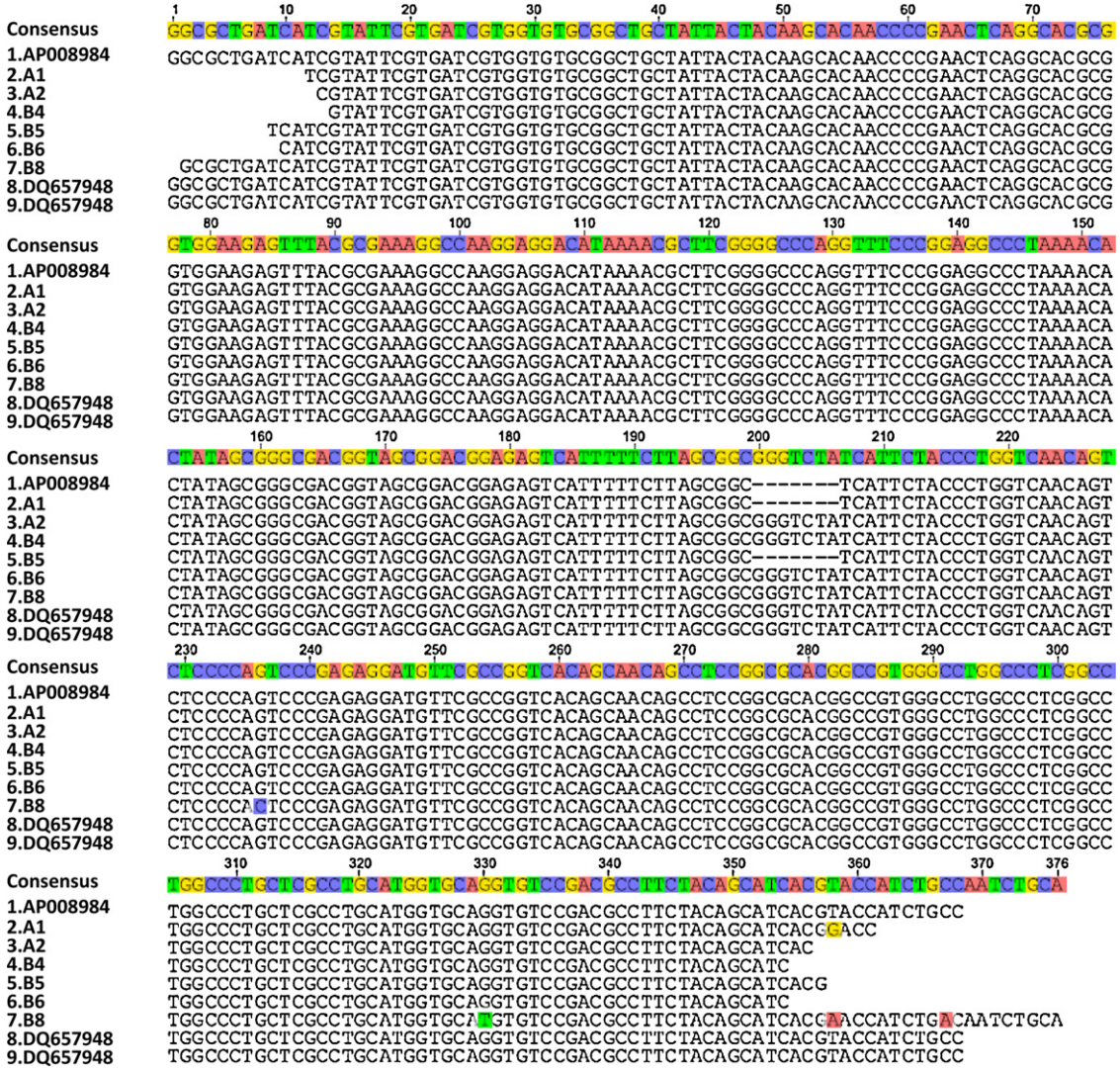

Fig. 5. DNA sequence alignments of nested PCR products amplified with primers specific for ORF-26. A1 and A2: KHV of wild common carp obtained in group 1; B4, B5, B6 and B8: KHV of wild common carp obtained in group 2; AP008984: KHV-J; DQ177346: KHV-U; DQ657948: KHV-I.

genotype 34 samples from similar geographic distribution, it also grouped those samples into two groups, Asian and European. This suggests that KHV may have originated from a common ancestor and diverged independently in two different geographic locations. KHV samples from outside Asia have more variation from KHV-J. This suggests KHV was independently introduced or emerged in the respective geographic locations. Another possibility is that KHV may have derived from an innocuous virus of *C. carpio* or another of the more than 2000 species of cyprinid fishes, escaping from a long-standing equilibrium with its host and adapting via increased virulence under conditions of aquaculture, where susceptible hosts are in abundant supply, constantly renewed, and transported worldwide (Aoki et al., 2007). If the latter speculation is true, it is also possible that KHV variants may have existed in wild common carp before the emergence of clinical KHV disease. There were two events of KHV-associated mortality reported in wild common carp in western countries, USA in 2004 (Grimmett et al., 2006) and Canada in 2007 (Garver et al., 2010). KHV outbreaks in carp populations may have resulted from environmental changes and accumulated mutations of the viral genome. One of the unique characteristics of all herpesviruses is the ability to establish a latent infection, which can be reactivated under various stressful conditions. It has demonstrated that KHV does indeed become latent following initial infection (Eide et al., 2011b; St-Hilaire et al., 2005). All carp captured from either the flood pond or Barnyard Springs showed no signs of illness, which suggests that KHV latency is present in wild common carp in Oregon. All koi obtained from the fish supplier in Oregon were also healthy with no signs of illness. This study suggests that KHV latent infection is present in both wild common carp and ornamental koi in Oregon. Since KHV DNA identified in wild common carp has genetic features similar to both Asian and European groups, these KHV variants in the wild common carp may have originated from an early ancestor from an innocuous virus of *C. carpio* or another of the more than 2000 species of cyprinid fishes.

Herpesviruses are known to be well adapted to their host and rarely cause significant disease. However, stress and environmental changes can cause reactivation from a latent infection. This raises the possibility that infection with low pathogenicity KHV may have been common in wild common carp before 1990, since very few KHV survey has been conducted in wild common carp populations (Uchii et al., 2009). This is the first investigation of KHV latent infection in healthy looking wild common carp from a location without the history of KHV infection. Many herpesviruses encode genes that can modulate the host immune response and evade the host immune defenses. Two immune suppressor genes, TNFR and IL-10,

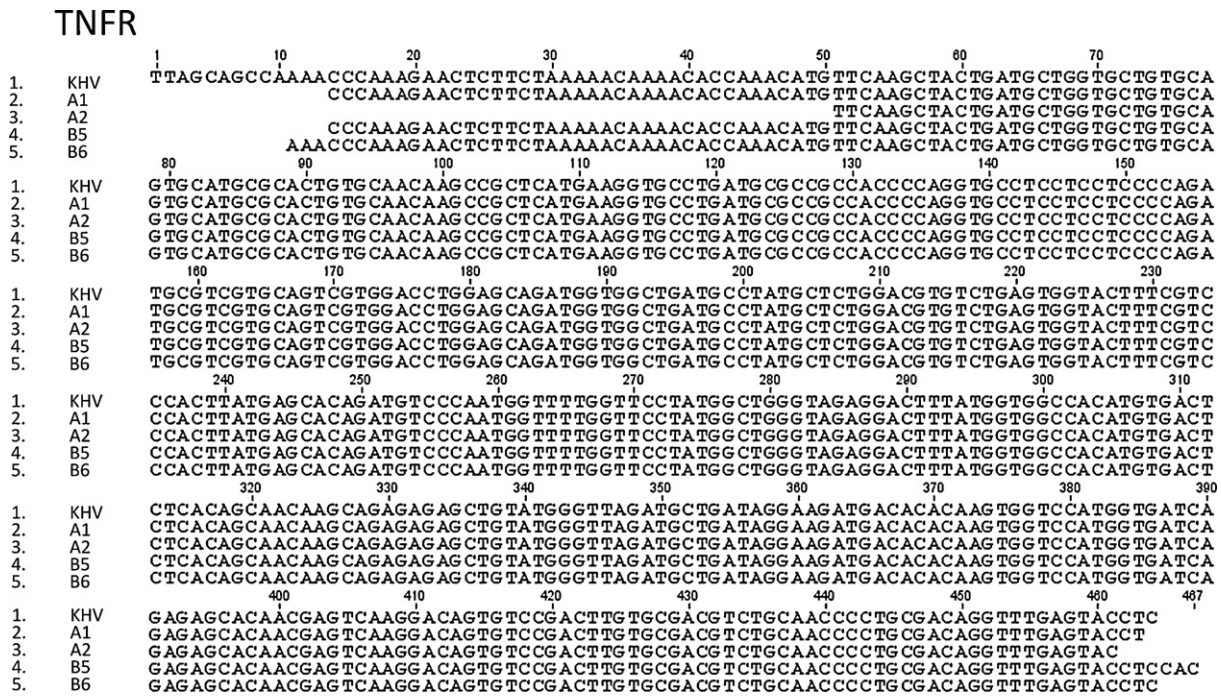

Fig. 6. DNA sequence alignments of nested PCR products amplified with primers specific for TNFR. A1 and A2: KHV of wild common carp obtained in group 1; B5 and B6: KHV of wild common carp obtained in group 2; AP008984: KHV-J; DQ177346: KHV-U; DQ657948: KHV-I.

are coded by the KHV genome. DNA sequence analysis of these two genes in wild common carp KHV did not identify any differences between the wild common carp KHV and reference KHV. This suggests that these immune modulating genes are conserved and may play important roles in the virus' ability to evade the host immune system.

KHV ORF-25 and ORF-26 are part of the ORF-25 family which encode six type I membrane glycoproteins. Type I proteins have a single transmembrane (TM) stretch of hydrophobic residues, with the portion of the polypeptide on the NH_2-terminal side of the TM domain exposed on the exterior side of the membrane and the COOH-terminal portion exposed on the cytoplasmic side. Most herpesvirus membrane glycoproteins are viral surface structure proteins and are important for virus entry, such as glycoprotein B and glycoprotein D, which serve as the major host receptor binding proteins during initial infection. Because of the 13 nt deletion in ORF-25 of KHV-U and KHV-I, it is 144 aa shorter than ORF-25 of KHV-J. The 7 nt deletion in ORF-26 only occurs in KHV-J. It will be interesting to know what effects these deletions have on virus entry or virulence of KHV. All wild common carp KHV DNA sequenced in this study have the 13 nt deletion in ORF-25, which suggests they have an ORF-25 with a function similar to that of KHV-U or KHV-I. Additionally, some wild common carp KHV have the 7 nt deletion in ORF-26, and have an ORF-26 function similar to that of KHV-J. In addition to deletion differences in ORF-25 and ORF-26, several single nucleotide mutations were observed in ORF-25–26 coding

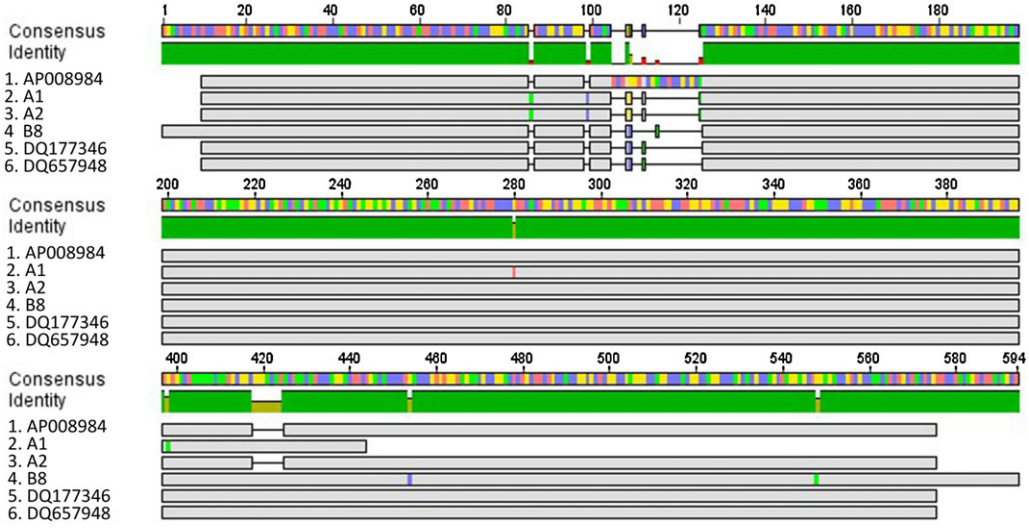

Fig. 7. DNA sequence alignments of nested PCR products amplified with primers specific for ORF-25 and ORF-26. The 13 nt deletion is near nt 120 relative to the reference strain AP008984. A1 and A2: KHV of wild common carp obtained in group 1; B8: KHV of wild common carp obtained in group 2; AP008984: KHV-J; DQ177346: KHV-U; DQ657948: KHV-I. Green bar: 100% identical. Green line: T insertion or mutation from G to T; blue: C insertion or mutation from G to C; orange line: mutation from G to A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sequences of KHV DNA from wild common carp (Fig. 7). Overall, these differences suggest that KHV detected in wild common carp is a variant of KHV, which may be less virulent compared to KHV isolates associated mortality.

KHV DNA detected in the ornamental koi has genetic features similar to KHV-J in ORF-25 and ORF-26 coding region. Koi were acquired from a fish supplier in Oregon where no KHV outbreak has been reported. This raises the possibility that these koi may have been exposed to a KHV variant that is less virulent. It is interesting that KHV DNA from ornamental koi is similar to KHV-J, but different from KHV-U or KHV-I. Many koi in the USA ornamental fish trade have been acquired from Japan. It is possible that these ornamental koi originated from Japan and harbor a less virulent form of KHV which is similar to KHV-J.

During KHV latency, KHV DNA is detectable in white blood cells using both PCR and Southern blot (Eide et al., 2011a). However the previously described methods are not sufficient to obtain enough genetic material for DNA sequencing. Using nested PCR methods reported in this study, PCR products can be sequenced directly. This method can be used to detect KHV latency and differentiate the KHV strain. In addition, KHV detection rate in the white blood cells is much higher than the detection rate in a whole fish (Eide et al., 2011a). Therefore, white blood cells are the best sample for screening of KHV latent infections. The reason that only 4 of 10 WBC samples were positive from group 1 (A1–A10) of wild common carp is that several blood samples were coagulated before arriving to the lab. Therefore, a second group of wild common carp was acquired (group 2, B1–B14). Overall, this study demonstrated that latent KHV in wild common carp can be detected and sequenced using nested PCR methods. These data suggest that it is possible that KHV may have existed in wild common carp before the recognition in late 1990s. Whole genome sequence of the wild common carp KHV may identify genes that could shed light on the pathogenesis and evolution of KHV.

Competing interests

The authors declare that they have no competing interests.

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