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Detection of koi herpesvirus (KHV) in healthy cyprinid seed stock

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ABSTRACT: Koi herpesvirus (KHV) disease is a lethal disease in common carp, an important food fish in Asian countries, the seed of which is used in restocking programs for freshwater fishery management. We inspected apparently healthy seed stock of common carp *Cyprinus carpio* L. and Siberian crucian carp *Carassius auratus* for the presence of KHV using PCR-based diagnostic tests as a part of a stock enhancement program from 2009 to 2010 in Korea. Consequently, KHV was detected from 24 of 232 inspections with yearly detection percentages of 5.2% in 2009 and 15.5% in 2010 using PCR primer sets for TK or *Sph*I-5 as recommended by the OIE Manual of Diagnostic Tests for Aquatic Animals. Results indicate that the *Sph*I-5 primer set was slightly more sensitive than the TK primer set, as shown by a higher detection rate. To determine the genotype of the KHV strains detected in this study, ORF40-specific PCR amplification was conducted, and the PCR products from 6 samples showed 100% nucleotide sequence identity with a Japanese strain (GenBank accession number AP008984) but not with US (DG657948) and Israeli strains (DG177346). This report conclusively demonstrated the presence of KHV in externally healthy seed of common carp and Siberian crucian carp, indicating a possible risk that subclinically infected seed stock can be released with a potential threat to wild populations.

KEY WORDS: Carp · *Cyprinus carpio* · *Carassius auratus* · Cyprinid herpesvirus 3 · PCR · ORF40

INTRODUCTION

The common carp *Cyprinus carpio* L. has a broad geographical distribution and is found in natural freshwater bodies around the world (Rahel 2007). This species is also intensively cultivated as an important human food source in Europe and Asia, and the ornamental variety, koi carp, is also highly valued. In Korea, common carp and Siberian crucian carp *Carassius auratus* are the main carp species found in the wild and used in aquaculture. Korean local governments buy and release cultured carp seed to enhance aquatic resources.

Siberian crucian carp have been domestically cultured in East Asia including Korea. This species differs from crucian carp *C. carassius* in Europe but shares an identical scientific name with goldfish *C. auratus* (Takada et al. 2010). Crucian carp in Korea are Siberian crucian carp, as they have been reported as *C. auratus* based on morphological and genetic analyses (Nam et al. 1989, Jung 2013).

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Marine and inland stock enhancement by releasing aquatic animals raised in hatcheries has been considered a potential management tool for increasing production and profitability of fisheries and meeting the demand for food requirements for an ever-increasing human population (Bartley et al. 2006). In Korea, hatchery-reared carp seed, especially common carp and Siberian crucian carp, have been released into the environment for the purpose of restoring the indigenous fish species and replenishing severely depleted fisheries in inland ranching programs.

Concerns about unfavorable consequences of hatchery-reared seed releases such as the spread of pathogens, competition for food, and cannibalism, as well as genetic deterioration of wild stocks, have been increasing within the framework of aquatic animal health management (Fushimi 2001). Subasinghe (2005) also pointed out that movements of live aquatic animals and animal products (broodstock, seed, and feed) have accelerated the accidental spread and incursion of diseases into new populations and geographic regions, for example, through movements of hatchery-produced stocks. Thus special efforts have been required to prevent disease spread due to the release of cultured seed into the ecosystem, in terms of aquatic animal health management (Blankenship & Leber 1995).

To prevent transmission of infectious agents by releasing hatchery-raised seed into the wider environment as part of stock enhancement programs, the Korean government has been conducting inspections of all hatchery-reared seed stock with emphasis on notifiable infectious diseases, since the Aquatic Animal Disease Control Act came into force in 2008 in Korea. For carp, there are 3 specific diseases of concern, i.e. spring viremia of carp, koi herpesvirus (KHV) disease, and infectious pancreatic necrosis. Release of seed stock infected with any of the listed pathogens is prohibited by the Act, and the government can force farmers or local governments to limit the seed within a restricted area to prevent the spread of the pathogen. Transmission of KHV by release of cultured carp may threaten wild fish since KHV can cause mortality in wild carp (Terhune et al. 2004, Grimmett et al. 2006, Uchii et al. 2009).

KHV, also called cyprinid herpesvirus 3 (CyHV-3) (Waltzek et al. 2005, Haramoto et al. 2007), belongs to the family *Alloherpesviridae* and is genetically similar to the other cyprinid herpesviruses carp pox virus (CyHV-1) and goldfish hematopoietic necrosis virus (CyHV-2), but is distinguished from channel catfish virus (IcHV-1) and ranid herpesvirus 1 (RaHV-1) (Waltzek et al. 2005). KHV is most lethal and highly contagious to carp, with disease signs that include skin ulcers, excess mucus, and hemorrhages in the fins (OIE 2013). Previously, KHV was known to be pathogenic only to common carp. When goldfish, grass carp *Ctenopharyngodon idella*, crucian carp, and other species were exposed to KHV or co-cultured with severely KHV-infected common and koi carp, no clinical signs were observed (Gilad et al. 2002, Perelberg et al. 2003, Ronen et al. 2003). However, more recent studies indicate that KHV is not restricted to koi and common carp; for example, KHV was observed to propagate and induce cytopathic effect in cells derived from goldfish and silver carp *Hypophthalmichthys molitrix* as well as in koi and common carp cells (Davidovich et al. 2007). Although some species are not susceptible to KHV disease, they may act as carriers or reservoirs of KHV and transmit infection to co-habiting sensitive wild species. Indeed, Fabian et al. (2013) demonstrated that KHV of carp can be transferred to naïve carp by cohabitation, as well as to wild cyprinids such as roach *Rutilus rutilus* and tench *Tinca tinca*, and other non-cyprinid fishes.

Detection of KHV can be undertaken by isolation in cell culture, serological methods, and/or PCR-based methods. PCR-based detection is the most sensitive method and appears to be more reliable than virus isolation in cell culture; serological assays are not yet fully validated (Haenen et al. 2004). PCR analysis is essential to confirm the disease, although cell culture and serological assays can detect the virus (OIE 2013). Although PCR-based methods have been developed to detect KHV (Gilad et al. 2002, Gray et al. 2002, Yuasa et al. 2005), their sensitivity is not sufficient to detect the virus in carrier fish (Bergmann et al. 2006). Thus the more sensitive nested PCR and *in situ* hybridization have also been used (Bergmann et al. 2009).

Of further interest is the existence of various genotypes of KHV. According to Aoki et al. (2007), an ancestral KHV diverged to form 2 lineages, i.e. Japan and Israel/USA, based on full-length sequence analysis. Bigarré et al. (2009) reported a third intermediate lineage, which is aligned with neither the Japan nor the Israel/USA genotypes, in fish samples from Poland and the Netherlands based on the alleles of 3 domains targeting an intergenic region between open reading frame (ORF) 29, ORF30, and ORF133. Indeed, the sequence analysis of 2 other regions between ORF29 and ORF31 (marker I) and near the start of ORF133 (marker II) indicated that all Indone-

sian isolates are a new intermediate lineage (Sunarto et al. 2011). The Indonesian lineage is more closely related to the Asian than the European lineage. The study of genetic relationships based on ORF40 demonstrated that Austrian isolates share 100% nucleotide sequence identity with the Japanese strain of KHV (Marek et al. 2010).

Here we report the detection of KHV from healthy seed stock of cyprinids to be released as part of a stock enhancement program in Korea from 2009 to 2010 and the genotype analysis of the detected KHV based on sequence analysis of the ORF40 gene.

MATERIALS AND METHODS

Sampling

Common carp and Siberian crucian carp were collected from 2009 to 2010 by local governments for laboratory analysis. In each case, 30 or 50 fish were delivered live in a plastic bag to the laboratory and sacrificed by an overdose with the anesthetic MS-222. Sizes of common carp or Siberian crucian carp ranged from 6 to 10 cm and 4 to 8 cm, respectively, and all of them appeared clinically healthy. Samples of brain, spleen, and kidney were removed aseptically and pooled (10 fish pool^{-1}) in a tube to obtain sufficient tissue (50 mg) for DNA extraction.

Spleen and kidney were chosen since gill, kidney, and spleen are known to be the organs in which KHV is most abundant during the course of overt infection (Gilad et al. 2004). Brain was chosen since the OIE (2013) recommended including intestine and encephalon when testing sub-clinical or apparently healthy fish by PCR-based methods.

DNA extraction

Approximately 50 mg of tissues were used to extract total DNA using a High Pure PCR template preparation kit (Roche). Briefly, 200 µl of tissue lysis buffer and 40 µl of Proteinase K were added to the tissue and incubated for 1 h or overnight at 55°C. After adding binding buffer and incubating for 10 min at 70°C, 100 µl of isopropanol were added and the preparation was transferred to a spin column and centrifuged for 1 min at 6300 × *g*. The column was washed with 500 µl of inhibitor removal buffer once and with wash buffer twice by centrifugation at 6300 × *g* for 1 min. The DNA was eluted with 50 µl of pre-warmed elution buffer. The quality and concentration of the eluted DNA were determined by agarose gel electrophoresis and a spectrophotometer (NanoVue, GE Healthcare), respectively. The DNA concentration was adjusted to 100 ng µl^{-1} for PCR.

Detection of KHV by PCR and sequencing

Diagnosis of KHV was based on the detection of KHV DNA in tissue by PCR using TK (Bercovier et al. 2005) and *Sph*I-5 (as modified by Yuasa et al. 2005) primer sets (OIE 2013). The primer sequences, PCR conditions, and product sizes are listed in Table 1.

Table 1. PCR primers and conditions used for koi herpesvirus detection in this study

Protocol	Primer sequence (5′–3′)	PCR step	PCR conditions			Product size (bp)	Reference
			Temp. (°C)	Time (min)	Cycles (n)		
TK	Forward	Initial denaturation	94	5	1	409	OIE (2013)
	GGGTTACCTGTACGAG	Amplification	95	1	40		
	Reverse		55	1			
	CACCCAGTAGATTATGC		72	1			
		Final elongation	72	10	1		
*Sph*I-5	Forward	Initial denaturation	94	1	1	292	OIE (2013)
	GACACCACATCTGCAAGGAG	Amplification	94	0.5	40		
	Reverse		63	0.5			
	GACACATGTTACAATGGTCGC		72	0.5			
		Final elongation	72	7	1		
ORF40	Forward	Initial denaturation	95	15	1	445 (J type),	Aoki et al.
	TTGAAACGGTGAGGCAGCCAT	Amplification	95	0.5	40	458 (U type),	(2007)
	Reverse		60	0.5		460 (I type)	
	CAACTGCAACATACCGTCAAG		72	1			
		Final elongation	72	10	1		

Each reaction contained 2.5 µl 10× ExTaq buffer, 2 µl dNTP mixture (2.5 mM each), 0.125 µl Hot Start ExTaq polymerase (Takara), 1 µl each forward (10 µmol μl^{-1}) and reverse (10 µmol μl^{-1}) primers, and 1 µl template DNA in a final volume of 25 µl. In each case, positive and negative samples were added to validate the PCR.

The PCR products were visualized using Multi-Image ®II (Alphainnotech) following electrophoresis on 1.5% agarose gel containing ethidium bromide (0.6 µg ml^{-1}).

To ensure the detection of KHV, all PCR products were sequenced and the nucleotide sequences analyzed using the BLAST program to compare with the previously reported KHV sequences in the NCBI data bank. Briefly, the PCR products were purified using a gel purification kit (Gel SV kit; GeneAll) and cloned into the TOPO-TA vector (Invitrogen). After plasmid DNA extraction using a Plasmid SV mini kit (GeneAll), PCR products were sequenced in both directions using T7 and Sp6 primers.

Sequence analysis of the ORF40 region of KHV isolates

Subsequent PCR targeting ORF40 was carried out to identify the genotype of the detected KHV in 2010. Each reaction contained 2.5 µl 10× ExTaq buffer, 2 µl dNTP mixture (2.5 mM each), 0.125 µl Hot Start ExTaq polymerase (Takara), 1 µl each ORF40 forward (10 µmol μl^{-1}) and ORF40 reverse (10 µmol μl^{-1}) primers (Table 1), and 2 µl template DNA (50 µg ml^{-1}) in a final volume of 25 µl. The oligonucleotide primer pairs used in this study amplify the partial ORF40 gene from the US (GenBank accession number DQ657948), Israeli (DQ177346), and Japanese (AP008984) KHV types, with sizes of 458, 460, and 445 bp, respectively (Aoki et al. 2007).

The PCR products were purified, cloned, and sequenced by the method described above. Nucleotide sequences from 6 isolates including 1 derived from common carp (KORCC1012) and 5 derived from Siberian crucian carp (KORCrC1015, -1017, -1020, -1021, -1024) were aligned and compared with the US, Israeli and Japanese KHV types available in the GenBank/EMBL nucleotide database using the program Genetyx v. 7 (Genetyx).

RESULTS

According to the local government survey from 2009 to 2010, 3441 common carp and 26 440 Siberian crucian carp were released for the stock enhancement program. The released Siberian crucian carp accounted for 88.5% of the total carp (Table 2). Geographically, common carp were released mainly in the southern part of Korea including Gyungnam, Jeonnam, Gyungbuk, and Jeonbuk provinces, while Siberian crucian carp were released in the northern part including Jeonbuk and Gyunggi provinces (Table 2).

Out of 232 (116 in each year) inspections, 24 cases tested positive for KHV (Table 3). The positive percentage increased from 5.2% in 2009 to 15.5% in 2010. Of the 24 positive results, 6 (out of 62; 12.9%) were common carp and 16 (out of 170; 9.4%) were Siberian crucian carp. With respect to the geographical distribution of the KHV-positive fish farms, 5 were in Gyungnam, 4 in Choongbuk, 3 in Gyunggi, and 1 in Jeonnam province. The number of positive cases was slightly higher using *Sph*I-5 primers than TK primers for both common carp and Siberian crucian carp (Table 4).

Nucleotide sequence analysis of the ORF40 PCR products (445 bp) was undertaken on 6 isolates. The nucleotide sequences were all identical to the Japan-

Table 2. Number of juvenile cyprinids released (×1000) from 2009 to 2010 for stock enhancement in Korea. GN: Gyungnam, GB: Gyungbuk; JN: Jeonnam; JB: Jeonbuk; CN: Chungnam; CB: Chungbuk; GW: Gangwon; GG: Gyonggi

Species	Year	Province									Total
		GN	GB	JN	JB	CN	CB	Busan	GW	GG	
Common carp	2009	656	225	283	278	20	277	–	–	–	1739
	2010	783	289	420	210	–	–	–	–	–	1702
	Sub total	1439	514	703	488	20	277	–	–	–	3441
Siberian crucian carp	2009	1168	1055	1298	3001	891	1092	110	2486	2322	13423
	2010	2663	1699	670	2827	920	792	163	1394	1889	13017
	Sub total	3831	2754	1968	5828	1811	1884	273	3880	4211	26440
Total	5270	3268	2671	6316	1831	2161	273	3880	4211	29881	

Table 3. Koi herpesvirus detection in juvenile cyprinids used for stock enhancement in Korea

Year		Common carp	Siberian crucian carp	Total	Percentage
2009	Released	1739	13423	15162	
	Inspected	30	86	116	0.8
	PCR Positive	2	4	6	5.2
2010	Released	1702	13017	14719	
	Inspected	32	84	116	0.78
	PCR Positive	6	12	18	15.5

Table 4. PCR results using different primer sets for koi herpesvirus detection in juvenile cyprinids from 2009 to 2010 for stock enhancement in Korea. Province abbreviations are given in Table 1; farm names are abbreviated to preserve confidentiality

Species	Sampling date (yyyy.mm)	Location		PCR result	
		Province	Farm	TK	*Sph*I-5
Common carp	2009.09	GN	EH	–	+
Common carp	2009.10	GN	CE	+	+
Common carp	2010.06	GN	CE	+	+
Common carp	2010.08	GN	CE	+	+
Common carp	2010.06	GN	HI	+	–
Common carp	2010.08	GN	GI	–	+
Common carp	2010.10	GN	GI	–	+
Common carp	2010.09	GG	IR	+	+
Siberian crucian carp	2009.08	CB	CB	+	+
Siberian crucian carp	2009.09	GN	EH	+	+
Siberian crucian carp	2009.09	GN	CE	+	+
Siberian crucian carp	2009.10	GN	CE	+	+
Siberian crucian carp	2010.06	GN	HI	–	+
Siberian crucian carp	2010.08	GN	JI	–	+
Siberian crucian carp	2010.08	JN	HC	+	–
Siberian crucian carp	2010.08	CB	JG	+	+
Siberian crucian carp	2010.08	CB	UG	+	+
Siberian crucian carp	2010.08	CB	CP	+	+
Siberian crucian carp	2010.09	CB	CP	+	+
Siberian crucian carp	2010.07	GG	JSI	+	+
Siberian crucian carp	2010.08	GG	JSI	+	–
Siberian crucian carp	2010.08	GG	SJ	–	+
Siberian crucian carp	2010.09	GG	SJ	–	+
Siberian crucian carp	2010.09	GG	IR	+	+
No. positive/total				17/24	21/24

ese strain of KHV (AP008984) but mismatched with the US (DQ657918) and Israeli strains (DQ177346) (Fig 1).

DISCUSSION

In this study, common carp and Siberian crucian carp were inspected for the presence of KHV before release as part of a stock enhancement program. Using PCR tests (OIE 2013), KHV was detected in tissue samples from apparently healthy seed stock of cultured common carp and Siberian crucian carp, with a detection rate of 15.5% in 2010, similar to the detection rate of 15% (6/41 fish) in common carp and Israeli carp *Cyprinus carpio nudus* reported by Kim et al. (2010) for fish farms that had previously undergone mass mortalities between 1999 and 2007. In addition, KHV was detected in heavily infected brood stock of koi carp farms in Korea (Gomez et al. 2011). However, ours is the first report of KHV detected in apparently healthy carp in Korea and in any Siberian crucian carp in Korea.

Although the tested seed was mainly Siberian crucian carp, which is known to be resistant to KHV disease, this is not insignificant because carrier Siberian crucian carp could potentially spread the pathogen to other wild fish, as has been reported for goldfish, which are also known to be resistant to KHV disease (Bergmann et al. 2010). El-Matbouli & Soliman (2011) also reported that KHV propagated in goldfish and that the goldfish acted as carriers since KHV was detected by PCR in specific pathogen free carp after co-culturing with infected goldfish.

These results raise serious concerns, as common carp and Siberian crucian carp are cultured together in most domestic fish farms in Korea, where hybrids between common carp and Siberian crucian carp can be observed. Although mass mortality due to KHV has not been reported in domestic cultured Siberian crucian carp, their hybrids may be susceptible. Hedrick et al. (2006) reported that goldfish and carp × goldfish hybrids did not show severe clinical signs following infection with KHV, whereas Bergmann et al. (2010) reported that 35% mortality occurred in koi × goldfish hybrids and 100% mortality in koi × crucian carp hybrids.

Results from our study indicate that Siberian crucian carp co-cultured with KHV-susceptible species such as common carp are at risk of infection. Although any such transmission may only result in

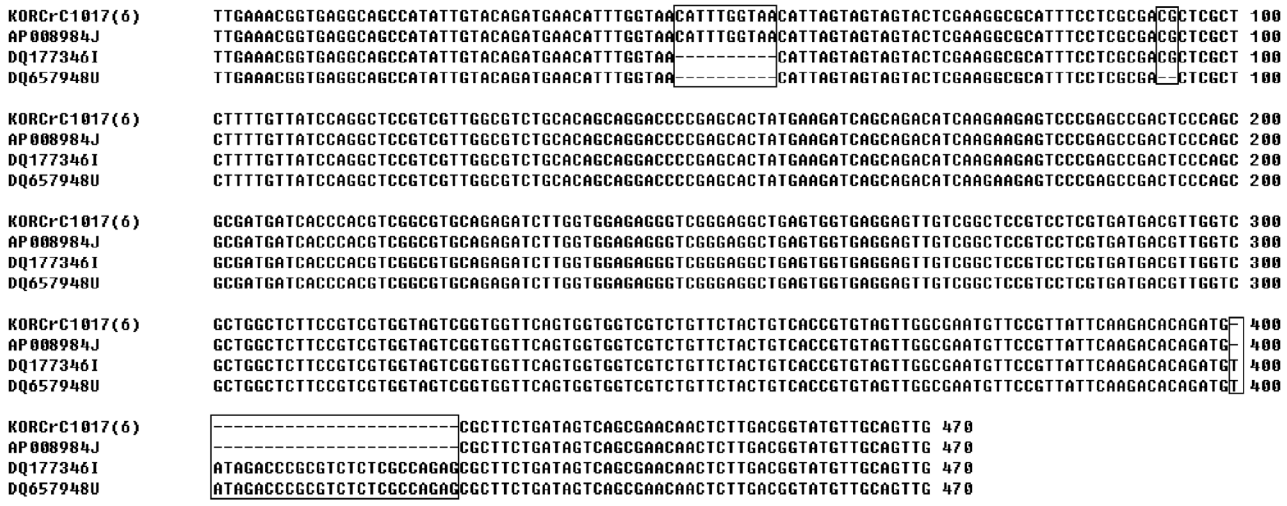

Fig. 1. Comparison of the KHV ORF40 nucleotide sequences of the representative sample detected in this study (KORCrC1017) with Japanese (J), US (U) and Israeli (I) genotypes (AP008984, DQ657948 and DQ177346, respectively)

sub-clinical infection, it may be persistent, resulting in the Siberian crucian carp being a secondary carrier. In addition, as juvenile common carp and Siberian crucian carp have been used as live feed in domestic fish farms producing species such as the golden mandarin fish *Siniperca scherzeri*, the potential risk for expansion of the KHV host range cannot be ruled out.

While several reports have indicated that single-step PCRs have some limitations for the detection of low-level KHV infections (Bergmann et al. 2009, Monaghan et al. 2014), we have demonstrated that the PCR tests used were able to detect KHV in apparently healthy or carrier fish. In this study, the number of positive cases was slightly higher using *Sph*I-5 primers than TK primers.

Nucleotide sequence analysis of the KHV isolates detected in this study demonstrated that they belong to the Japanese genotype. The first outbreaks of KHV were reported in Germany in 1997 and 1998 (Bretzinger et al. 1999). KHV was also detected from koi and common carp in Israel and the USA in 1998 (Hedrick et al. 2000). Since then it has been identified in various regions from at least 30 countries including the USA (Hedrick et al. 2000), Israel (Ronen et al. 2003), Japan (Sano et al. 2004), Poland (Bergmann et al. 2006), and other countries in Europe (Haenen et al. 2004) and Southeast Asia (Kurita et al. 2009). Comparisons of KHV isolates from different geographical areas have shown that KHV isolates from Japan, USA, and Israel have 100% identity in the nucleotide sequences of the DNA polymerase and MCP genes (Ishioka et al. 2005), and KHV isolates from Poland and Germany are also similar to those viruses (Antychowicz et al. 2005). In addition, the polypeptides of KHV isolates from various regions were also found to be similar (Gilad et al. 2002, 2003). Marek et al. (2010), using primer sets targeting the ORF40 region, showed that KHV derived from Japan, USA, and Israel produced amplicons of 445, 458, and 460 bp, respectively. These primer sets were useful for the classification of KHV derived from various regions (Marek et al. 2010).

In this study, 6 of the KHV isolates were analyzed and shown to be identical to the Japanese type (GenBank accession number AP008984) based on an analysis of the KHV ORF40 region as suggested by Marek et al. (2010). Therefore, the KHV detected in this study is likely derived from the Japanese strain. Similarly, Bigarré et al. (2009) reported that most KHV recently isolated in the Netherlands were of the Japanese genotype, and Marek et al. (2010) reported that 15 Austrian isolates were 100% identical to the Japanese strain. Marek et al. (2010) suggested that an experimental infection using KHV isolates with various genetic characteristics is needed to analyze the effect of viral genetic type on viral pathogenicity and host mortality. As the KHV isolates in this study were all detected in apparently healthy carp, experimental infectivity trials to compare pathogenicity of these isolates with KHV isolates from disease outbreaks should prove interesting.

In conclusion, we detected KHV in clinically healthy carp seed by PCR using TK or *Sph*I-5 primer sets, and the genotype was shown to be the Japanese type based on an analysis of ORF40 gene sequences.

Although they are apparently healthy and have uncertain susceptibility to KHVD, KHV-infected Siberian crucian carp may transmit the virus to other, more susceptible fish inhabiting the same water system. It is possible that KHV may be present as low-level infections, and remain undetected, in a range of freshwater species. Thus further studies on KHV distribution in freshwater species in Korea together with identification of genotypes is needed, particularly in the waterways where carp are released as part of the re-stocking program.

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