Importation of CyHV-2-infected goldfish into the Netherlands

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ABSTRACT: Cyprinid herpesvirus 2 (CyHV-2) is known as the causative agent of herpesviral haematopoietic necrosis in goldfish Carassius auratus auratus. However, the virus has also been detected in Prussian carp C. gibelio and crucian carp C. carassius from European and Asian countries. To prevent spread of the causative virus to other areas, investigation of the risk factors of spread of this virus is important. In this study, 8 batches of goldfish imported into the Netherlands by airfreight from Asia and the Middle East were investigated for the presence of the virus. CyHV-2 DNA was detected by PCR in the pooled kidneys of 4 of the 8 imported goldfish batches, of which 1 was from a CyHV-2 disease case at a Dutch importer’s quarantine facility. Sequence analysis of the CyHV-2 strains from this study and from previous reports showed that there were at least 6 different lengths in the mA region, resulting in tentatively at least 4 genotypes. Virus isolation was positive for only 1 (Amsterdam Schiphol-1 [AMS-1]) of the 8 samples. It was shown that the AMS-1 isolate was highly virulent to Ryukin goldfish after 10^3.3 TCID₅₀ fish⁻¹ intraperitoneal injection. The viral titre of the AMS-1 isolate for goldfish fin cells at several temperatures was similar to that of a Japanese CyHV-2 isolate. Our results prove that one of the routes of spread of various CyHV-2 strains is through the global trade of apparently healthy infected goldfish.

KEY WORDS: Cyprinid herpesvirus 2 · Goldfish · Ornamental fish · International trade · Disease spread · Risk factor · Genotype

INTRODUCTION

The disease herpesviral haematopoietic necrosis (HVHN) has caused great economic losses to goldfish Carassius auratus auratus aquaculture in Japan since it was initially reported in 1992 (Jung & Miyazaki 1995). The causative agent is cyprinid herpesvirus 2 (CyHV-2), which belongs to the genus Cyprinivirus of the family Alloherpesviridae according to the rules for nomenclature of the International Committee on Taxonomy of Viruses (Pellett et al. 2012). It has a double-stranded DNA genome of ~290 kbp in length. The virus is now recognized as a major pathogen of goldfish, not only in Japan but also in the USA (Grof et al. 1998, Goodwin et al. 2006), Taiwan (Chang et al. 1999), Australia (Stephens et al. 2004), New Zealand (Hine et al. 2006), the UK (Jeffery et al. 2007), France (Boitard et al. 2016) and Switzerland (Giovannini et al. 2016). Although it was thought that this virus showed virulence only to goldfish, mass mortalities caused by CyHV-2 infection were reported in Prussian carp, a synonym of gibel carp C. gibelio, in China and in the Czech Republic (Daněk et al. 2012, Wang et al. 2012). Furthermore, this virus was also detected in high amounts associated with disease and mortalities in wild gibel carp populations in the Netherlands (Haenen et al. 2016) and in wild and farmed crucian carp C. carassius populations in Italy (Fichi et al. 2013, 2016). These reports suggest that this virus has been spreading by movement of diseased fish and also that CyHV-2 has the potential to infect other species of the genus Carassius.
In general, it is well known that piscine viruses spread to other areas by movement of diseased or carrier fish and virus-contaminated eggs (Bootland & Leong 2011, Borzym et al. 2014, Rimmer et al. 2015). To prevent the spread of CyHV-2 to noninfected areas, it is essential to study the invasion route of the virus. In this study, therefore, 7 batches of goldfish from Asia and the Middle East were sampled for CyHV-2 testing directly after arrival at the Schiphol international airport, Amsterdam, The Netherlands, and 1 batch of diseased goldfish (shubunkin) was sampled at an importer’s quarantine facility after import from the Middle East. Schiphol airport is an important import and transfer station for the global ornamental fish trade (Dey 2016). We detected CyHV-2 DNA in 4 batches (3 in apparently healthy goldfish directly from Schiphol and 1 in the diseased shubunkin from the importer’s quarantine facility), and the virus was isolated from 1 of the apparently healthy 7 batches only. In experimental infection, the pathogenic properties to goldfish of the CyHV-2 isolate were investigated. Our findings demonstrated that international trade of apparently healthy goldfish is a risk of spread of virulent strains of CyHV-2.

MATERIALS AND METHODS

History of samples

Between December 2014 and November 2015, 8 batches of goldfish Carassius auratus auratus, imported into the Netherlands from China, Singapore, Hong Kong and Israel by airfreight, were used for this study. In this time frame, 7 batches of these goldfish were imported to specific Dutch importers into Europe via Amsterdam Schiphol Airport on various dates and kindly offered for testing by these importers via the Netherlands Food and Consumer Product Safety Authority. For further testing, 2 live fish were randomly selected from each goldfish batch, according to sampling instructions from the fish diseases laboratory at Wageningen Bioveterinary Research (WBVR), Wageningen University & Research (UR), The Netherlands. The 2 live goldfish per batch were packed in their original transport water in double plastic bags with oxygen and transported to WBVR. Additionally, 1 batch originated from a serious shubunkin disease case at a quarantine facility of a Dutch ornamental importer a few days after arrival of the fish from Israel. These fish were transported as described in this paragraph. Then, the goldfish batches were each separately euthanized with an overdose of 2-phenoxy-ethanol and dissected using disposable gloves and sterilized cutting gear and tables per batch, and their kidneys were pooled. The kidney samples were put into eight 1.8 ml sterile cryotubes without any addition and stored at approximately −80°C. These 8 tubes with pooled kidneys were sealed and transported to the Tamaki Laboratory of the National Research Institute of Aquaculture (NRIA), Japan, under frozen conditions. Details of the samples of this study are shown in Table 1.

Analysis of imported samples

Detection of CyHV-2 viral DNA

For the specific detection of CyHV-2, conventional PCR targeting the CyHV-2 helicase gene described

<table>
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<tr>
<th>Sample no. (given name)</th>
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<th>Clinical status</th>
<th>CyHV-2 DNA</th>
<th>Virus isolation</th>
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<td>Singapore</td>
<td>Apparently healthy</td>
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<tr>
<td>2</td>
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<td>China</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3 (AMS-3)</td>
<td>16 Dec 2014</td>
<td>Israel</td>
<td>Apparently healthy</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>13 Jan 2015</td>
<td>Singapore</td>
<td>Apparently healthy</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>15 Jan 2015</td>
<td>Hong Kong</td>
<td>Apparently healthy</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6 (AMS-6)</td>
<td>27 Jan 2015</td>
<td>Singapore</td>
<td>Apparently healthy</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>24 Feb 2015</td>
<td>Singapore</td>
<td>Apparently healthy</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8 (AMS-8)</td>
<td>27 Nov 2015</td>
<td>Israel</td>
<td>Diseased*</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Shubunkins, kept at 18°C, showed a mortality of 10% d−1 since arrival. Clinical signs included lethargy, surfacing, swollen belly, red gill region, congested and pale gill lamellae and epithelium with cell proliferation and congested and enlarged kidney.
by Waltzek et al. (2009) was used. Total DNA of the kidneys was isolated using a Gentra Puregene Tissue Kit (Qiagen). The DNA template for the positive control was extracted from the supernatant of CyHV-2 (Saitama-1 [SaT-1])-infected goldfish fin (GFF) cells (Li & Fukuda 2003, Ito et al. 2013) using InstaGene™ Matrix (Bio-Rad Laboratories). The PCR reaction mixture used was TaKaRa Ex Taq® Hot Start Version (TaKaRa) according to the manufacturer’s protocol. A thermocycling profile was performed according to the published protocol (Waltzek et al. 2009). Four PCR-positive samples, named Amsterdam Schiphol-1 (AMS-1), AMS-3, AMS-6 and AMS-8, were used for subsequent experiments.

**Analysis of three genomic regions**

The 3 genome regions, helicase (Waltzek et al. 2009), mA and mB (Boitard et al. 2016), of the viral DNA were sequenced. To investigate the enlarged mA region of the analyzed samples, a primer was designed on neighbouring mA genomic fragments as follows. The primer sequence for amplification of the upstream region of the oPVP382 primer was 5’-GTT GGT TTT GAT TCA TTT TTA TTT TA-3’, named 382up3. This primer was designed based on the sequence of ST-J1, a Japanese CyHV-2 isolate (GenBank accession number JQ815364), and SY-C1, a Chinese CyHV-2 isolate (GenBank accession number KM200722). The PCR reaction mixture used was TaKaRa Ex Taq® Hot Start Version (TaKaRa) according to manufacturer’s protocol. Amplicons obtained by the procedure described in this paragraph were visualized by electrophoresis in an ethidium bromide-stained 1.5% agarose gel. The PCR products were purified using a MinElute PCR Purification Kit (Qiagen) and directly sequenced in both orientations with the primers or inner sequencing primers used for amplification. In case the bands were faint, bands of the expected size were collected from the gel and purified using a MinElute Gel Extraction Kit (Qiagen). Then, the purified DNAs were ligated into the qCR4-TOPO vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen) and cloned into Escherichia coli strain DH5α. Plasmids from at least 5 independently derived clones were extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced in both orientations, after which consensus sequences were determined. For all nucleotide sequencing, a BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and ABI PRISM® 3100 Genetic Analyzer (Life Technologies) were used. The DNA sequences of SaT-1, AMS-1, AMS-3, AMS-6 and AMS-8 were submitted to DDBJ/EMBL/GenBank under accession numbers LC202016 to LC202029 (see Figs. 2–4 and Figs. S1–S3 in the Supplement at www.int-res.com/articles/supp/d126p051_supp.pdf). These sequence data were compared with those of ST-J1, the Japanese CyHV-2 isolate (GenBank accession number JQ815364), and those of SY-C1, the Chinese CyHV-2 isolate (GenBank accession number KM200722).

**Virus isolation**

The GFF cell line and standard Ryukin Takafumi (SRTF) cell line (Ito et al. 2013) from the fin of the Ryukin variety of goldfish were used in this study. Cultures of these cell lines were maintained in minimum essential medium (MEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio) and antibiotics (100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹) at 25°C. GFF and SRTF cells were cultured in CELLSTAR Standard Cell Culture Flasks (Greiner Bio-One) and Collagen Type I Coated Flasks (Iwaki), respectively.

Homogenates of individual kidneys were prepared with a glass homogenizer, using approximately 50 times the volume of sterile MEM. After centrifugation of the suspension for 10 min at 4°C and 400 × g, the supernatant was harvested and sterile filtered (0.45 µm). One day after fresh passages of GFF and SRTF cells in 25 cm² flasks, the culture medium was removed from the monolayer, and 500 µl of the kidney homogenate was added and absorbed at 25°C for 1 h, after which fresh medium supplemented with 10% FBS was added to the flask. Flasks were incubated at 25°C. The cells were observed every day for the occurrence of cytopathic effect (CPE) over a period of 14 d. After 14 d, a new blind passage was done accordingly and incubated at 25°C for another 14 d. When no CPE had occurred after 2 blind passages, the samples were considered negative in virus isolation.

To isolate CyHV-2 from the imported goldfish samples, virus isolation was performed also by in vivo passage in goldfish. The goldfish variety Edonishiki was used for in vivo passages. These Edonishiki were bred from CyHV-2-free broodstock at the Tamaki Laboratory, NRIA. Edonishiki goldfish (weight 14.8 g and length 7.5 cm, on average) were divided into 9 groups of 4 fish. All injected fish were anaesthetized using 2-phenoxyethanol diluted 1:1000 (v/v) with well water. The fish in 8 of 9 groups were each inoculated intraperitoneally with 100 µl of the super-
natant of one of the kidney homogenates of the 8 imported goldfish batches. For this injection, the homogenates of the kidneys were used and were prepared as described in the previous paragraph, without filtration, but after centrifugation at 400 × g for 10 min at 4°C. The fish in the last group were inoculated intraperitoneally with 100 µl of MEM as a negative control. All 9 goldfish groups were kept in 60 l tanks with flow-through freshwater at 24.0°C (23.5 to 24.0°C), fed a commercial diet once a day and observed for disease and mortalities daily until 28 d after inoculation. In all experimental groups, the kidneys of each dead fish and each surviving fish were tested individually by virus isolation at 25°C using GFF and SRTF cells and by PCR targeting the CyHV-2 helicase gene (Waltzek et al. 2009), as described in ‘Detection of CyHV-2 viral DNA’ above.

Replication of AMS-1 isolate in cell line

Multiplication in the goldfish cell lines of the SaT-1 and AMS-1 isolates was assessed by serial 10-fold dilutions (10² to 10⁻⁵) in each of the 8 well columns of 96-well microplates on GFF cell lines at 4 different temperatures, 15, 20, 25 and 30°C. To ensure reliable results, the 2nd and 4th passages of the virus culture supernatant were tested for virus titre. Titration was performed by the endpoint dilution method, and the results were expressed as TCID₅₀ ml⁻¹ (Reed & Muench 1938).

Experimental infection

The reference virus used for the experimental infection was the CyHV-2 SaT-1 isolate (Ito et al. 2013). The CyHV-2 AMS-1 isolate which was isolated in this study was used for an experimental infection of goldfish. The 2 isolates were propagated in 5 subsequent passages in GFF cells, using 75 cm² flasks (Greiner Bio-One) at 25°C. Aliquots of the cell culture supernatant containing CyHV-2 were placed in 3.6 ml cryotubes (Nunc®, Thermo Fisher Scientific) and stored at −85°C until use. One aliquot was thawed, inoculated in serial 10-fold dilutions (10⁰ to 10⁻⁵) in each of the 8 well columns of 96-well microplates, and seeded with a freshly sub-cultured GFF cell suspension. The microplates were incubated at 25°C for 21 d to measure the titre of the viral suspension stored for use in subsequent experimentally induced infections in goldfish. The goldfish variety Ryukin was used for the experimental injection challenge. Ryukin were bred from CyHV-2-free broodstock at the Tamaki Laboratory, NRIA. Eggs were disinfected with iodophor (200 mg l⁻¹, 15 min) immediately after fertilization. The hatched fry were fed water fleas Daphnia sp. until 14 to 21 d after hatching and were subsequently fed commercial pellets (Saki-Hikari®, Kyorin) and kept at approximately 18 to 24°C. All stages were maintained in clean well water to prevent infections.

The Ryukin goldfish (weight 7.1 g and length 6.7 cm, on average) were divided into 5 groups of 20 fish, 1 group per tank. Eighty Ryukin goldfish (2 x 2 groups) were intraperitoneally inoculated with 100 µl of the cell culture supernatant containing CyHV-2 SaT-1 or AMS-1 at 10⁰.₅ and 10¹.₃ TCID₅₀ fish⁻¹, respectively. Twenty Ryukin goldfish (1 group) were intraperitoneally inoculated with cell culture medium only, as a negative control. All fish groups were kept in 60 l flow-through aquaria with well water (0.6 l min⁻¹) and were fed a commercial diet (Saki-Hikari®, Kyorin) once a day. Disease and mortality were recorded for 28 d. The water temperature was kept at 25.8°C (24.7 to 26.2°C). The surviving goldfish were euthanized by an overdose of 2-phenoxyethanol prior to dissection. All fish experiments and all handling of fish were in accordance with Guidelines for Animal Experimentation of the NRIA, Fisheries Research Agency, Japan. In all experimental groups, the kidneys of each dead fish and each surviving fish were tested individually by virus isolation using GFF cells and by CyHV-2 PCR to detect the virus, as described above in this section.

RESULTS

Clinical signs of samples

The clinical signs of the 8 batches of goldfish Carassius auratus in this study are shown in Table 1. The
goldfish in Samples 1 to 7 were apparently healthy. However, the shubunks in Sample 8, kept at 18°C, showed a mortality of 10% d⁻¹ since arrival. The gross pathological signs of the fish were lethargy, surfacing, swollen belly, red gill region, congested and pale gill lamellae and gill epithelium with cell proliferation, and congested and enlarged kidney.

**Analysis of imported samples**

**Detection of CyHV-2 DNA from goldfish kidney samples**

Results of the detection of CyHV-2 DNA from goldfish kidney samples in this study are shown in Table 1. The results of electrophoresis of PCR amplicons, using different primers, are shown in Fig. 1. CyHV-2 DNA was detected in Samples 1 (AMS-1), 3 (AMS-3), 6 (AMS-6) and 8 (AMS-8) by the conventional PCR method of targeting the helicase gene; the viral DNA was not detected in Samples 2, 4, 5 and 7. PCR amplicons of the mA region of AMS-1, AMS-3, AMS-6 and AMS-8 were ~500 bp. Several bands of PCR amplicons were observed in Samples AMS-3 and AMS-6 after electrophoresis. Although CyHV-2 DNA of the mA region was detected in AMS-8, the size of the amplicons was smaller than in the other PCR-positive samples. From the results of electrophoresis, the size of the amplicons of AMS-8 was judged to be ~300 bp. CyHV-2 DNA of the mB region was detected in the AMS-1, AMS-6 and AMS-8 samples at the expected size. We did not observe any specific PCR amplicon from Sample AMS-3.

![Fig. 1. (A) Detection of CyHV-2 DNA by PCR in kidney pools from goldfish imported into the Netherlands, using specific primers targeting the helicase gene (Waltzek et al. 2009). Arrow indicates the position of the 366 bp amplicon. (B) Detection of CyHV-2 DNA from the positive samples of the helicase gene by PCR using specific primers targeting the oPVP382+oPVP383 (mA) and oPVP384+oPVP385 (mB) regions (Boitard et al. 2016). Arrows indicate the positions of the 462 and 475 bp amplicons of the positive control (SaT-1 isolate) on the mA and mB regions, respectively. M: molecular weight marker (Gene Ladder 100, Wako); N: negative control; P: positive control, numbered lanes correspond to sample numbers shown in Table 1](image-url)
Analysis of three genomic regions

The helicase gene region (Waltzek et al. 2009) of the AMS-8 sample had 4 nucleotide deletions compared to the other 3 positive samples (AMS-1, AMS-3, and AMS-6) and to ST-J1, the Japanese CyHV-2 isolate (JQ815364), and SY-C1, the Chinese CyHV-2 isolate (KM200722). No difference was observed in the helicase gene region of other obtained CyHV-2 DNA samples (Fig. 2 and Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d126p051_supp.pdf).

Alignments of the mA and mB regions of the SaT-1, AMS-1, AMS-3, AMS-6 and AMS-8 samples are shown in Fig. 3 (Fig. S2 in the Supplement) and Fig. 4 (Fig. S3 in the Supplement). A few variations in the length of the mA region were observed among the analyzed CyHV-2 samples, since there were a few repeats of sequences in the region. The length of the mA region of SaT-1, AMS-1 and AMS-3 was 462 bp. The length of the mA region of AMS-6 was 471 bp. In contrast, the length of the mA region of AMS-8 was 297 bp. It was shown that there were a few misanneal-

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Isolates (Accession no.)

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Fig. 2. Aligned sequences of the site of the helicase region (Waltzek et al. 2009) in DNA of CyHV-2-positive samples. Numbers indicate nucleotide positions. Disagreements of nucleotides are indicated by dots. Annealing positions of primers are shown using arrows. ST-J1: Japanese CyHV-2 isolate; ST-C1: Chinese CyHV-2 isolate; SaT-1: Saitama-1; AMS: Amsterdam Schiphol
ing sites in the mA region of all 5 samples. The regions are shown as light green letters in Fig. 3. As a result of the PCR to target the enlarged mA region, the amplicon was not observed only in AMS-3 after electrophoresis. It was shown that there were 2 annealing sites of oPVP382 in the enlarged mA region of AMS-6, as in SY-C1, the Chinese CyHV-2 isolate. The regions in light blue in Fig. 3 are the annealing sites. Moreover, there was a misannealing site in the enlarged mA region of AMS-6 and SY-C1.

The length of the mB region of SaT-1, AMS-1, ST-J1 and SY-C1 was 475 bp, and the lengths of the mB region of AMS-6 and AMS-8 were 473 and 474 bp, respectively. It was shown that there was a deletion and a substitution after the base position of 444 (Fig. 4 and Fig. S3 in the Supplement).

Virus isolation

No virus was isolated from any of the pooled kidney samples from the 8 batches of imported goldfish directly, after 2 blind passages in GFF and SRTF cells (Table 1). However, the virus was isolated from 1 of 8 pooled kidney samples following in vivo passage in goldfish as follows.

A goldfish inoculated with the supernatant of the kidney homogenate of AMS-1 (Sample 1) died 7 d post-inoculation. Subsequently, all (4 of 4) inoculated fish from this group died by 12 d post-inoculation. CyHV-2 DNA was detected by PCR, and CyHV-2 was isolated from the kidneys of all dead goldfish in GFF and SRTF cells (Table 1). This isolate was used for experimental infection and multiplication in the GFF cell line. At 21 d post-inoculation, no mortality was observed in the groups of goldfish inoculated with the SaT-1 isolate at doses of $10^{0.3}$ and $10^{1.3}$ TCID$_{50}$ fish$^{-1}$, respectively. Both groups of goldfish inoculated with the AMS-1 isolate at doses of $10^{0.3}$ and $10^{1.3}$ TCID$_{50}$ fish$^{-1}$ showed cumulative mortalities of 100%. CyHV-2 DNA was detected from the kidneys of all dead fish in all virus-inoculated groups. The virus was re-isolated from the kidneys of all dead fish in all infected groups. CyHV-2 DNA was detected from surviving fish in the high-dose SaT-1 group. However, no CPE was observed in cultures inoculated with samples from surviving fish in this group. There was no mortality in the negative control goldfish, and viral DNA was not detected from the kidneys of these goldfish. No virus was isolated from any fish in the negative control group.

DISCUSSION

CyHV-2 has been reported as a causative agent of HVHN of goldfish (Jung & Miyazaki 1995, Stephens et al. 2004, Goodwin et al. 2006, Jeffery et al. 2007). Recently, the virus has also been detected from Prussian carp or gibel carp (Daněk et al. 2012, Wang et al. 2012, Haenen et al. 2016) and crucian carp (Fichi et al. 2013, 2016) from European and Asian countries. These reports suggest that this virus has been spreading by movement of infected fish and also that CyHV-2 has the potential to infect other species of the genus Carassius.

In this study, we investigated whether CyHV-2 was detected from batches of goldfish imported into the Netherlands from countries outside the European Union. CyHV-2 was detected in 4 of 8 batches of imported goldfish, 2 from Singapore and 2 from Israel; in the latter case, 1 batch had already been stocked into the quarantine facility of the fish importer. Our results prove that one of the routes of spread of various CyHV-2 strains is through the global trade of apparently healthy infected goldfish. Since CyHV-2
Fig. 3. Aligned sequences of the site of primers oPVP383 and oPVP382up3 (enlarged mA) (Boitard et al. 2016 and this study) of CyHV-2 DNA isolates. Numbers indicate nucleotide positions. Disagreements of nucleotides are indicated by dots. Annealing positions of primers are shown using arrows. FR: French CyHV-2 isolate; other isolate IDs as in Fig. 2.
in Australia was first identified in 2003 at a goldfish farm, Stephens et al. (2004) suggested that CyHV-2 was introduced with imported ornamental fish. Moreover, CyHV-2 was found at retail outlets and farms and in several populations of wild goldfish in Australia (Becker et al. 2014). In addition, Rimmer et al. (2015) reported that dwarf gourami iridovirus, a strain of infectious spleen and kidney necrosis virus, had been detected from some imported live ornamental fish species, such as red tiger oscar *Astronotus ocellatus* and dwarf gourami *Trichogaster lalius*. This study adds to previous findings that demonstrate that the international trade of ornamental fish assists in the spread of fish pathogens.

In this study, Sample 8 was sampled from a severe disease case of CyHV-2 in shubunkin goldfish, but the virus was not replicating at all in homologous cells (GFF and SRTF), even after *in vivo* passage. However, the virus was isolated from apparently healthy goldfish (Sample 1), only after passage *in vivo*. Giovannini et al. (2016) also reported detection of CyHV-2 from apparently healthy goldfish in Switzerland.

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Usually, imported goldfish are sold to retailers, from which private owners might stock them into indoor aquaria or their garden ponds, which are mostly closed facilities and therefore of low risk. However, in cases where a pond is connected with

### Table 2. Titration of 2 CyHV-2 isolates (SaT-1 and AMS-1) in goldfish fin cells, measured after the 2nd and 4th passages of virus isolation

<table>
<thead>
<tr>
<th>Incubation temp. (°C)</th>
<th>2nd passage Titre (TCID&lt;sub&gt;50&lt;/sub&gt; ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>4th passage Titre (TCID&lt;sub&gt;50&lt;/sub&gt; ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SaT-1</td>
<td>AMS-1</td>
</tr>
<tr>
<td>15</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>10&lt;sup&gt;1.6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;1.8&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2.2&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>10&lt;sup&gt;2.5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### Table 3. Results of detection of CyHV-2 DNA by PCR and re-isolation of the virus from the individual kidneys of experimentally inoculated fish. Values are given as number of positive fish per number of fish tested. SaT-1: Saitama-1; AMS-1: Amsterdam Schiphol-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Infectious dose (TCID&lt;sub&gt;50&lt;/sub&gt; fish&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Cumulative mortality (%)</th>
<th>Dead fish PCR</th>
<th>Virus re-isolation PCR</th>
<th>Surviving fish PCR</th>
<th>Virus re-isolation PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaT-1</td>
<td>10&lt;sup&gt;2.3&lt;/sup&gt;</td>
<td>100</td>
<td>20/20</td>
<td>20/20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;1.3&lt;/sup&gt;</td>
<td>85</td>
<td>17/17</td>
<td>17/17</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>AMS-1</td>
<td>10&lt;sup&gt;2.3&lt;/sup&gt;</td>
<td>100</td>
<td>20/20</td>
<td>20/20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;1.3&lt;/sup&gt;</td>
<td>100</td>
<td>20/20</td>
<td>20/20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>0/20</td>
<td>0/20</td>
<td>–</td>
</tr>
</tbody>
</table>
open water, wild stocks of susceptible fish species such as gibel carp might become infected with CyHV-2.

Regarding the helicase gene region of analyzed CyHV-2 DNA samples in our study, only 4 nucleotide deletions of AMS-8 compared with the others were found. However, the detected CyHV-2 DNA samples from imported goldfish (AMS-1, AMS-3, AMS-6 and AMS-8) had different sequences compared to so-far-reported strains, ST-J1 and SY-C1, in the mA and mB regions (Boitard et al. 2016). The mA regions of the SY-C1 (358 bp) and ST-J1 (432 bp) strains were smaller than those of the CyHV-2 strains of SaT-1 (reference CyHV-2 strain in this study; 462 bp), AMS-1 (462 bp), AMS-3 (462 bp) and AMS-6 (471 bp). Remarkably, the length of the mA region of AMS-8 was 297 bp. Boitard et al. (2016) reported that the sequence of the mA region of a French strain revealed an exact size of 295 bp. Therefore, from these results and previous studies, it shows that there are at least 6 different lengths in the mA region of CyHV-2 strains. The enlarged mA region of the AMS-3 strain was not amplified by PCR. Although a few primers were designed on neighbouring 382up3 and tested for amplification, the specific amplicon was not obtained from the AMS-3 DNA sample (data not shown). Consequently, it was supposed that the AMS-3 strain had a different sequence compared to sequences of the other CyHV-2 strains in neighbouring oPVP382up3.

Regarding the mB region of the analyzed CyHV-2 DNA strains, there were a few deletions and substitutions among ST-J1, SY-C1, SaT-1, AMS-1 and AMS-6. In addition, the mB region of the AMS-3 strain which was detected from goldfish imported from Israel (according to the importer) was not amplified by PCR. It was suggested that the AMS-3 strain had different sequences compared to the other CyHV-2 strains and for the primer of the oPVP384 or oPVP385 regions. Full genomes of CyHV-2 are so far published for only 2 isolates (Davison et al. 2013, Li et al. 2015), with a genome size of approximately 290 kbp. Sequence analyses of the genome of cyprinid herpesvirus 3 (CyHV-3), which belongs to the same family, Alloherpesviridae, have shown that CyHV-3 can be divided into 2 major genotypes (Aoki et al. 2007, Bigarré et al. 2009, Kurita et al. 2009). Similarly, CyHV-2 may be divided into plural genotypes. As mentioned in the previous paragraph, the length of the mA region among CyHV-2 varies, which suggests that there are various genotypes in CyHV-2. Based on the length of the mA region, CyHV-2 can be tentatively divided into 4 major genotypes, i.e., with lengths of the mA region of approximately 300, 360, 430 and 470 bp (Fig. 3 and Fig. S2 in the Supplement at www.int-res.com/articles/suppl/d126p051_supp.pdf).

With regard to the virulence of CyHV-2 isolates to goldfish and gibel carp by experimental infections, some reports were published (Jung & Miyazaki 1995, Wang et al. 2012, Ito et al. 2013, Xu et al. 2013, Ito & Maeno 2014). In this study, it was shown that the AMS-1 isolate was highly virulent to Ryukin goldfish after intraperitoneal injection.

Since the AMS-1 isolate originated from Singapore, which is a tropical country, the isolate may have faced higher temperatures for a longer period than the Japanese CyHV-2 isolate in Japan. Therefore, we presumed the optimal temperature for multiplication of the AMS-1 isolate might be higher than that of the SaT-1 isolate. However, in this study, no remarkable differences in the titre were observed between both isolates at 4 different temperatures. The titres of the 2nd passage samples of both isolates tended to be higher than those of the 4th passage samples. Some previous studies report that continuous propagation of the CyHV-2 in cell culture had been problematic (Jeffery et al. 2007, Wang et al. 2012, Xu et al. 2013). Although we reported that we could sustainably propagate CyHV-2 using fish cell lines derived from goldfish (Ito et al. 2013), we also confirmed that serial passage of CyHV-2 in cell lines resulted in a gradual decrease of infectivity (data not shown). Hence, the results of this study do not contradict the results of previous reports.

Previously, Ito & Maeno (2014) revealed that cumulative mortalities of the 15, 20, 25 and 30°C groups were 10, 90, 90 and 60%, respectively. Therefore, the temperature range of 20 to 25°C is considered optimal for CyHV-2 infection in goldfish (Ito & Maeno 2014). However, Danék et al. (2012) described that massive mortalities of Prussian carp (= gibel carp) associated with CyHV-2 in the upper Elbe basin were observed between 16.1 and 20.5°C. These reports suggest the possibility that the optimal temperature of the disease varies according to the virus strain, the genotype of CyHV-2 and the host fish species. However, Haenen et al. (2016) reported a massive gibel carp mortality related to CyHV-2 infection at 20 to 25°C. Therefore, further studies are needed to analyze the genotypes of CyHV-2 and the methods to discriminate genotypes. Furthermore, it is important to further characterize the different genotypes of CyHV-2 isolates for virulence.

Since 2011, reports on outbreaks or detections of CyHV-2 in cultured and wild Prussian carp have increased in Asia and Europe (Danék et al. 2012,
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