# Mass mortality events associated with cyprinid herpesvirus 2 (CyHV-2) infection in wild Prussian carp Carassius gibelio in the Netherlands, and molecular biology of virus strains

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RESEARCH ARTICLE

# Mass mortality events associated with cyprinid herpesvirus 2 (CyHV-2) infection in wild Prussian carp *Carassius gibelio* in the Netherlands, and molecular biology of virus strains

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## Abstract

In 2011 and 2015, four mass mortalities of Prussian carp (*Carassius gibelio*) were observed in a recreational freshwater lake and open freshwater in the western part of the Netherlands. Cyprinid herpesvirus 2 (CyHV-2) infection was suspected in these cases, based on presumptive gross diagnosis. To elucidate the cause of the mass mortalities diagnostic PCR assays were performed for CyHV-2, based on the *helicase* gene. Furthermore, the viral isolates were genotyped by sequencing the enlarged marker A and marker B sequences. Diagnostic PCR revealed that three of four samples were positive for CyHV-2, indicating these three mass mortalities were associated with CyHV-2 infection. The marker A sequence from one of the isolates found in this study was identical to those from different locations such as Asia and Middle East, suggesting a link among the isolates. This is the first detailed report on mass mortalities of Prussian carp associated with CyHV-2 infection in natural aquatic environments in the Netherlands. Since 2015, additionally, in total three CyHV-2 associated outbreaks of Dutch Prussian carp were seen in 2016 and 2020. These outbreaks in Prussian carp from lakes and open water suggest that the virus has been spreading in natural freshwaters in the Netherlands.

KEYWORDS
CyHV-2, cyprinid herpesvirus 2, goldfish, mass mortality events, natural waters, Prussian carp

## 1 | INTRODUCTION

Cyprinid herpesvirus 2 (CyHV-2), a member in the family *Alloherpesviridae*, is the causative agent of herpesviral haematopoietic necrosis (HVHN) in goldfish (*Carassius auratus auratus*) and Prussian carp (*C. gibelio*) (Daněk et al., 2012; Jung & Miyazaki, 1995). CyHV-2 is highly transmissible and causes mass mortalities in goldfish and Prussian carp, and cumulative mortality rates of these two fish species may exceed 90% (Ito & Maeno, 2014; Daněk et al., 2012). Since CyHV-2 was first officially reported in Japan, cases associated with this viral infection have been observed all over the world (Thangaraj et al., 2021). The virus was detected not only from fish in aquaculture but also in fish from natural waters such as lakes and rivers, possibly due to illegal release of goldfish infected with CyHV-2 into natural waters (Becker et al., 2014). Currently, most of CyHV-2 associated mortalities were reported from fish farms and data from natural environment are still limited.

In 2011 and 2015, mass mortalities of Prussian carp were experienced in the western part of the Netherlands. CyHV-2 infection was suspected in these cases based on presumptive gross diagnosis. To elucidate the cause of these mass mortalities, diagnostic PCR assays were performed based on the *helicase* gene of CyHV-2 (Waltzek et al., 2009). Furthermore, the viral isolates were genotyped by sequencing the enlarged marker A (mA) and marker B (mB) sequences (Boitard et al., 2016; Ito et al., 2017). In the Netherlands, in 2016 and 2020, a total of three CyHV-2 newer associated mass mortalities of Dutch Prussian carp were diagnosed (Wageningen Bioveterinary Research or WBVR, own

  

findings). Strains of CyHV-2 from these cases were not studied in this paper, but their clinical pathology is reflected on in the discussion.

## 2 | MATERIALS AND METHODS

### 2.1 | Fish

When mass mortalities were observed in 2011 and 2015 in Prussian carp in a recreational freshwater lake and in open freshwater in the western part of the Netherlands, live, moribund or freshly dead Prussian carp were collected at these sites for diagnostics (Table 1). The Prussian carps were killed and were dissected for necropsy, including investigation for bacterial isolations and presence of ecto- and endo-parasites. Tissue samples were taken from the gills and the internal organs (spleen, heart and kidney) for virus isolation, quantitative PCR (for diagnosis) and PCR (for genotyping).

### 2.2 | Diagnostic laboratory tests at WBVR

The organs were pooled per batch of fish and processed to a 10% (w/v) suspension. Virus isolation was performed on FHM- (fathead minnow) and EPC- (Epithelioma papulosum cyprini) cells (EURL, 2021). In parallel, pooled gills and kidneys from each case of specimens for qPCR/PCR were fixed in EtOH (96–99%), and genomic DNA was isolated using the Qiagen DNA/Tissue Mini-Kit (Qiagen, Hilden, Germany). The qPCRs for CyHV-3 (Gilad et al., 2004) and CyHV-2 (Goodwin et al., 2006) were, respectively, performed and analysed on ABI 7500 Fastsystems (Applied Biosystems, Massachusetts, United States). The inoculated agar plates for bacteriology were incubated during 7 days at 22°C.

### 2.3 | Polymerase chain reactions for genotyping CyHV-2 isolates at FRA

Conventional PCRs were performed for the *helicase* gene, mA and mB sequences (Ito et al., 2017). The reactions were performed using primer sets for the helicase (CyHV2HelF and CyHV2HelR; Waltzek et al., 2009), mA (oPVP383 and oPVP382; Boitard et al., 2016) and mB sequence (oPVP384 and oPVP385; Boitard et al., 2016) as described by Ito et al. (2017). The reverse primer for mA sequence, oPCP382, originally developed by Boitard et al. (2016), did not amplify the target DNA fragment very well so that additional reactions were performed using a primer set, oPCP383 and oPVP383up3 (5′-TAAAATAAAAATGAATCAAA ACCAAC-3′), which amplifies the larger DNA fragment encompassing the mA target region (enlarged mA). All the amplified DNA fragments were sequenced. A commercially available software, Geneious Prime® ver. 2022.0.1, was used for cleaning up the sequencing results and calculating similarities of the gene sequences (Auckland, New Zealand).

### 2.4 | Phylogenetic analysis

A phylogenetic analysis was performed using the enlarged mA sequences obtained in this study along with others available in the NCBI GenBank database as described previously (Table 2; Soto et al., 2017). The *helicase* and mB sequences were not used for the sequence analysis because the sequences were nearly identical, and substitutions or insertions/deletions were rarely observed among the isolates.

## 3 | RESULTS

### 3.1 | Mass mortality events and clinical signs

During the four mass mortalities of Dutch wild Prussian carp in 2011 and 2015, high mortalities were recorded, (Haenen et al., 2016), whereby the water temperature in outbreaks 2–4 (positive for CyHV-2) varied from 20 to 25°C (Table 3). In the three positive cases of CyHV-2, their Ct values in the qPCR test of the $10^{-2}$ diluted organ suspensions were low (Ct ≤ 20), a sign of high amounts of the virus (Table 3).

**TABLE 1** Prussian carp (*Carassius gibelio*) mass mortalities in Dutch freshwaters in the Netherlands.

| Japan FRA ID | WBVR-NL ID | Sampling date | Location |
|---|---|---|---|
| NL-C.gibelio-1 | 11005487 | March 25th, 2011 | Wollebrand Recreational Lake, Honselersdijk, the Netherlands |
| NL-C.gibelio-2 | 11009701 | May 26th, 2011 | Open freshwater, Waddinxveen, the Netherlands |
| NL-C.gibelio-3 | 11015272 | August 29th, 2011 | Open freshwater, Polder de Noordplas, Zoetermeer, the Netherlands |
| NL-C.gibelio-4 | 15009145 | June 9th, 2015 | Wollebrand Recreational Lake, Honselersdijk, the Netherlands |

Abbreviations: FRA: Japan Fisheries Research and Education Agency; WBVR-NL: Wageningen Bioveterinary Research in the Netherlands.

**TABLE 2** List of the CyHV-2 DNA sequences from *Carassius* species, used for phylogenetic analysis. Only the marker A sequence was used for the analysis since the *helicase* gene and marker B sequences were highly conserved among the isolates so that these sequences were not suitable for phylogenetic analysis.

| Strain/isolate | Marker A[a] | Marker B[a] | Helicase[a] | Location | Year | Fish species[b] | Reference |
|---|---|---|---|---|---|---|---|
| ST-J1 | JQ815364.1 (7064–7593) | JQ815364.1 (213,940–214,414) | JQ815364.1 (126,318–126,683) | Japan | 1992 | Goldfish | Davison et al. (2013) |
| SaT-1 | LC202021.1 (1–560) | LC202026.1 (1–475) | LC202016.1 (1–366) | Japan | 1999 | Goldfish | Ito et al. (2013) |
| H. Fukuda | Not available | Not available | EU349287.1 (665–1030) | Japan | 1992–1993 | Goldfish | Waltzek et al. (2009) |
| JS2012 | Not available | Not available | KC245087.1 (665–1030) | China | 2012 | Prussian carp (Gibel carp) | Zhang et al. (2014) |
| SY-C1 | KM200722.1 (7002–7457) | KM200722.1 (213064–213,538) | KM200722.1 (125,605–125,970) | China | 2012 | Prussian carp (Gibel carp) | Li et al. (2015) |
| 1310[c] | KU199244.1 (28,409–28,851) | KU199244.1 (235,035–235,509) | KU199244.1 (147,438–147,803) | China | 2013 | Crucian carp | Zeng et al. (2016) |
| AMS-1 | LC202022.1 (1–560) | LC202027.1 (1–475) | LC202017.1 (1–366) | Singapore | 2014 | Goldfish | Ito et al. (2017) |
| AMS-3 | LC202025.1 (1–462) | Not available | LC202019.1 (1–366) | Israel | 2014 | Goldfish | Ito et al. (2017) |
| AMS-6 | LC202023.1 (1–569) | LC202028.1 (1–473) | LC202018.1 (1–366) | Singapore | 2015 | Goldfish | Ito et al. (2017) |
| AMS-8 | LC202024.1 (1–395) | LC202029.1 (1–474) | LC202020.1 (1–132) | Israel | 2015 | Goldfish | Ito et al. (2017) |
| RSD-PL | KX852452.1 (3–297) | Not available | Not available | Poland | 2016 | Goldfish | Panicz et al. (2019) |
| YZ-01[d] | L: MK260012.1 (6580–6972) R: MK260012.1 (280,232–280,624) | MK260012.1 (212,742–213,215) | MK260012.1 (125,211–125,576) | China | 2015 | Goldfish | Wang et al. (2021) |
| YC110907 | Not available | Not available | JQ067603.1 (332–697) | China | 2011 | Prussian carp (Gibel carp) | Pan et al., unpublished |
| SY[d] | L: KT387800.1 (7110–7552) R: KT387800.1 (282,212–282,654) | KT387800.1 (213,721–214,195) | KT387800.1 (126,128–126,493) | China | 2014 | Prussian carp (Gibel carp) | Lie et al., unpublished |
| CNDF-TB2015[d] | L: MN201961.1 (6987–7379) R: MN201961.1 (280,501–280,893) | MN201961.1 (212,869–213,342) | MN201961.1 (125,379–125,744) | China | 2015 | Crucian carp | Pan et al., unpublished |

(Continues)

TABLE 2 (Continued)

| Strain/isolate | Marker A[a] | Marker B[a] | Helicase[a] | Location | Year | Fish species[b] | Reference |
|---|---|---|---|---|---|---|---|
| NL-C.gibelio-2 | OR123886 (long) (1–631) OR123887 (short) (1–488) | OR123890 (1–429) | OR123883 (1–318) | Netherlands | 2011 | Prussian carp | This study |
| NL-C.gibelio-3 | OR123888 (1–514) | OR123891 (1–429) | OR123884 (1–318) | Netherlands | 2011 | Prussian carp | This study |
| NL-C.gibelio-4 | OR123889 (1–471) | OR123892 (1–429) | OR123885 (1–318) | Netherlands | 2015 | Prussian carp | This study |

[a]GenBank accession numbers and regions of the sequences used for the analyses.

[b]The scientific names are not shown in the list as the taxonomic identification of crucian carp is not well defined.

[c]The virus was reported as a Crucian carp *Carassius auratus* herpesvirus (CaHV), different from CyHV-2. However, due to its sequence similarity with CyHV-2, the viral genome sequence was included in this study.

[d]The mA sequence can be found in left (L) and right (R) arm, respectively, of the CyHV-2 whole genome sequences.

TABLE 3 Diagnostic findings of wild Prussian carp (*Carassius gibelio*) freshwaters in the Netherlands.

| Japan FRA ID | Mortality, length of fish | Year, month | Abnormalities as recorded by clinical signs (C), necropsy (N), parasitology (P) and bacteriology (B) | Virology v.i.[a]; qPCR KHV; qPCR CyHV-2, (Ct value CyHV-2)[b] |
|---|---|---|---|---|
| NL-C.gibelio-1 | High, 32 cm | 2011, 3 | C&N: Surfacing, light exophthalmos with blindness, rough and haemorrhagic skin and fins, congested firm ovary. P: No records on dead fish. B: Some multi-bacterial growth on agar. | Neg; Neg; Neg (No Ct) |
| NL-C.gibelio-2 | >2500 fish, 36 cm | 2011, 5 | C&N: Red skin and fins (fine haemorrhages), some small skin lesions. Congested spleen, haemorrhagic muscle, clear ascites, red anus. P: Externally many *Dactylogyrus & Gyrodactylus*, *Argulus* and ciliates (*Trichodina*, *Chilodonella*). B: Some multi-bacterial growth on agar. | Neg; Neg; Pos (Ct = 15) |
| NL-C.gibelio-3 | High, 25–35 cm | 2011, 8 | C&N: Red skin and fins (fine haemorrhages), haemorrhages in eyes, dull and easy loosening skin, gills pale with congested lamellae and hyperplastic epithelium, pale liver, petechial haemorrhages in swim bladder and muscle, red intestine, viscera, and ovary. P: Externally many *Dactylogyrus & Gyrodactylus*. B: Low, multi-bacterial growth on agar. | Neg; Neg; Pos (Ct = 18) |
| NL-C.gibelio-4 | >1400 kg, 15–40 cm | 2015, 6 | C: White mucus layer over the eyes, whole fish red of haemorrhages. N: not done. P & B: not tested. | Neg; Neg; Pos (Ct = 20) |

Abbreviations: FRA: Japan Fisheries Research and Education Agency; WBVR-NL: Wageningen Bioveterinary Research in the Netherlands.

[a]Virus isolation.

[b]In the $10^{-2}$ dilution of the fish organ suspension.

### 3.2 | PCR and sequencing reactions for genotyping CyHV-2 isolates

For the helicase and mB sequence of CyHV-2, DNA bands were amplified at the expected target sizes in the NL-C.gibelio 2 through 4, however, such DNA bands were not observed in the NL-C.gibelio 1 (Figure 1a,d). The DNA sequences of helicase and mB amplified in the NL-C.gibelio two through four were almost identical to those of the reference strain, SaT-1 (BLASTN search results: ≥99.7%).

Similar to the helicase and mB sequence, DNA bands were observed at the expected size for the enlarged mA sequence (Figure 1b,c). Notably, an extra band was amplified in the NL-C. gibelio 2 for the mA and enlarged mA (Figure 1b,c). For the enlarged mA sequence, both of the DNA fragments amplified in the NL- C.gibelio 2 (560 and 677 bp, Figure 1c) showed a high similarity to the mA sequence of the reference strain, but deletions of repeats were observed in the shorter DNA fragment (Figure 2, Supplemental Information 2–4).

## 4 | DISCUSSION

Haenen et al. (2016) reported the first mass mortalities of Prussian carp associated with CyHV-2 infection in natural freshwaters in the Netherlands in 2011, and subsequently in 2015, and then did not describe details. Such mortality events in Prussian carp from a recreational freshwater lake and open freshwater suggest that the virus has spread in the natural waters in this country. The susceptible species, Prussian carp has been found all over the Netherlands (Schiphouwer et al., 2014), so, the virus might be more spread than only in the Mid-Western part of this country. This was already partly confirmed, given the fact, that since 2015 three more mass mortalities of Prussian carp were diagnosed in the Netherlands, caused by CyHV-2: apart from one in 2016 in the Mid-West part (Ct non-diluted: 12), two subsequent CyHV-2 related mortalities were diagnosed in 2020 in one water area, in the North-Western part (WBVR, own findings) (Ct values non-diluted: 12, and 14, respectively). It is expected that more outbreaks will follow, also, because *C. gibelio* is present in all regions of the Netherlands (Schiphouwer et al., 2014).

Comparison of the mA sequences provided insight into the link of the CyHV-2 isolates. The mA sequence of NL-C.gibelio-3 is identical to those of AMS-1, −3, and SaT1 isolates (Figure 2, Supplemental Information 2). Among these isolates, AMS-1 and -3 were found in goldfish imported from Singapore and Israel, respectively, into the Netherlands in 2014 (Ito et al., 2017). In contrast, SaT1 was isolated from diseased Calico Goldfish in Japan over 20 years ago (Ito et al., 2013). The NL-C.gibelio-3 was detected from Prussian carp in the natural environment in the Netherlands (this study). Regardless of the locations and years of sampling, those CyHV-2 isolates share the identical mA sequence,

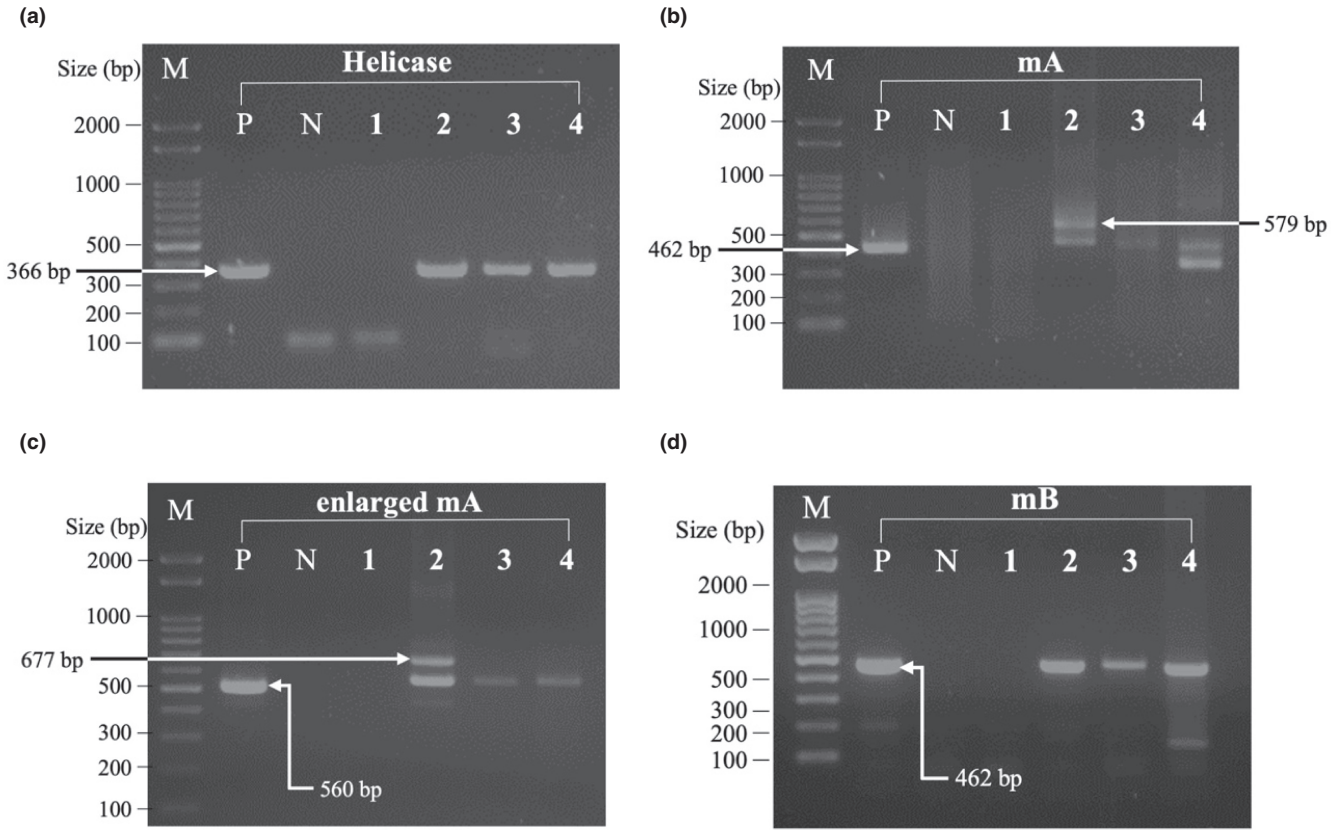


**FIGURE 1** Conventional PCR on CyHV-2 isolates from Prussian carp (*C. gibelio*) from the Netherlands. M: molecular weight marker; P: positive control; N: negative control; 1: NL-C.gibelio 1; 2: NL-C.gibelio 2; NL-C. 3; NL-C.gibelio 3; 4: NL-C.gibelio 4.

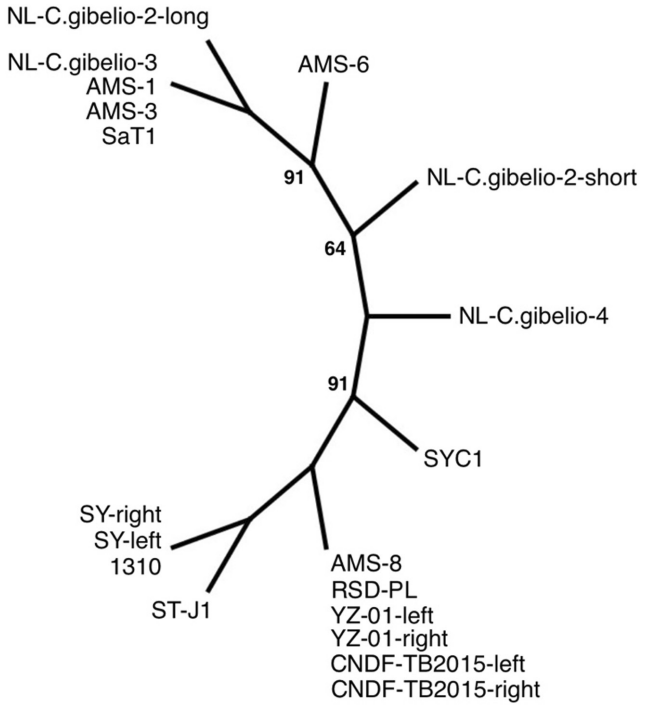


**FIGURE 2** Phylogenetic tree for the enlarged marker A sequences from various CyHV-2 isolates from *Carassius* species from various geographic regions.

suggesting that these isolates are somehow related to each other. One possible explanation is that the CyHV-2 isolate found in Japan over 20 years ago spread to other countries in Asia and the Middle East, and later entered the Netherlands. In any case, measures implemented at quarantine facilities at airports and ports may not be sufficient to prevent introduction of CyHV-2. This is partly due to the nature of CyHV-2; fish naturally infected with CyHV-2 usually do not show prominent external signs (Ito et al., 2013; Jung & Miyazaki, 1995). Furthermore, CyHV-2 can establish a persistent or latent infection (Wei et al., 2023). Therefore, introducing live goldfish from global imports has a high-risk regarding spreading CyHV-2, subsequently causing mass mortality events associated with this viral infection.

## AUTHOR CONTRIBUTIONS

**Takafumi Ito:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; project administration; supervision; data curation; resources; visualization. **Tomofumi Kurobe:** Writing – original draft; writing – review and editing; visualization; investigation; data curation; formal analysis; resources. **Jun Kurita:** Investigation; writing – review and editing; data curation; visualization; formal analysis; resources. **Olga Haenen:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; data curation; writing – original draft; visualization; resources. **Michal Voorbergen-Laarman:** Investigation; writing – review and editing; data curation; visualization; resources.

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## FUNDING INFORMATION

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## CONFLICT OF INTEREST STATEMENT

Not applicable.

## DATA AVAILABILITY STATEMENT

DNA sequence data are available in the GenBank Database (accession numbers: OR123883-OR123892). All the other data are within the paper and its supplemental information.

## CLINICAL TRIAL REGISTRATION

Not applicable.

## PATIENT CONSENT STATEMENT

Not applicable.

## PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

All the materials used in this paper are original. The DNA sequences used for phylogenetic analysis are freely available in a public database (GenBank Database, https://www.ncbi.nlm.nih.gov/genbank/) and those sequences are correctly cited in this paper.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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