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Seasonal Distribution of Cyprinid Herpesvirus 3 in Lake Biwa, Japan▽†

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The seasonal distribution of the cyprinid herpesvirus 3 (CyHV-3) in Lake Biwa, Japan, was investigated. CyHV-3 was distributed all over the lake 5 years after the first outbreak. The mean concentration of CyHV-3 in water showed annual oscillation, with a peak in the summer and a trough in winter. Our results suggested that CyHV-3 is present at high density in reductive environments, such as reed zones and turbid or eutrophic water.

A novel fatal disease of fish caused by cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus or carp interstitial nephritis and gill necrosis virus, which infects the common carp (*Cyprinus carpio carpio*) and ornamental koi (*Cyprinus carpio koi*) was reported at the end of the 1990s, and it has spread rapidly worldwide (13). In 2003, the first mass mortality in Japan was observed in Lake Kasumigaura, Ibaraki Prefecture (17), and the disease immediately spread all over the country.

Lake Biwa is the largest and oldest freshwater lake in Japan. It occupies 670 km^2, and the total length of the coastline is 241 km. The first and relatively small-scale mortality caused by CyHV-3 was observed in the autumn of 2003 in Lake Biwa, and mass mortality occurred in the following spring, in which more than 100,000 carp died (10). Thus, the disease is a great threat not only to the cultivation industry and koi collectors but also to the natural carp population. Carp is regarded as an ecological engineer that has an impact on freshwater ecosystems, and therefore, mass mortality will affect the entire lake ecosystem (10).

The pathogenesis and diagnosis of the disease have been studied intensively, but the dynamics of CyHV-3 has not been clarified. Recently, the major portal of CyHV-3 entry was reported to be fish skin (2), which means that infection via water is possible. Thus, to determine the method by which the virus spreads and to evaluate the infectious risk in the environment, one must clarify the CyHV-3 dynamics in the natural environment. In the present study, we surveyed the seasonal distribution pattern of CyHV-3 in Lake Biwa, Japan, using a quantitative method.

Concentration and quantification of viruses in environmental water. Twenty-two sampling sites were selected on the coastline of Lake Biwa, Japan (Fig. 1A) (see Table S1 in the supplemental material). Four liters of the lake surface water was collected in plastic tanks from these sites in 2007 in May to July, August, and November and in 2008 in February, May, June, August, October, and December (see Table S2 in the supplemental material). Although the first sampling was carried out from 15 May to 2 July 2007, hereafter, for simplicity, we refer to these as June 2007 samples. The water samples in plastic tanks were transported to the laboratory at room temperature. It took about 3 hours on average for transportation, and then the samples were stored at 4°C. To count the total number of viruslike particles and bacteria, 10-ml water samples were fixed with 2% formaldehyde and kept at 4°C. Samples were filtered onto 0.02-μm-pore-size Anodisc filters (Whatman) and stained with SYBR green I (Molecular Probes), and the enumeration of viruslike particles and bacteria was performed directly using an epifluorescence microscope (12). The other water quality parameters were measured on-site (Table 1) (see Table S2 in the supplemental material).

Virus concentration was performed on the next day of water sampling via a modified version of the cation-coated filter method (11; M. N. Honjo, T. Minamoto, K. Matsui, K. Uchii, H. Yamanaka, A. A. Suzuki, Y. Kohmatsu, T. Iida, and Z. Kawabata, submitted for publication). In brief, to estimate the CyHV-3 concentration, a known amount (1×10^7 viruslike particles $liter^{-1}$) of lambda phage was added to each water sample as an external standard. Viruses in the prefiltered lake water (4 liters) were trapped with cation (Al^{3+})-coated 0.45-μm HA electronegative filters (HAWP14250; Millipore, Japan). After the viruses were rinsed with 0.5 mM H_2SO_4, they were eluted with 200 ml of 1.0 mM NaOH. The concentrated viruses were then precipitated with 8% polyethylene glycol 6000 and 0.4 M NaCl at 4°C overnight and then centrifuged at 10,000 × *g* for 1 h. Viral DNA was extracted and purified by the phenol-chloroform method (16) and then further purified with the DNeasy blood and tissue kit (Qiagen, Germany). The final volume of the DNA solution was 100 μl. CyHV-3 and lambda DNA was quantified with a real-time TaqMan PCR (5; Honjo et al., submitted) using StepOnePlus real-time PCR systems (Applied Biosystems). For virus quantification, 5 μl and 2 μl of DNA solution were used as templates for CyHV-3 and lambda measurements, respectively, and the total volume of each reaction mixture was 20 μl. Three replicates were performed for each set.

The mean recovery yield of the lambda was 12.6% for all samples. Given that the recovery rate of CyHV-3 versus that of

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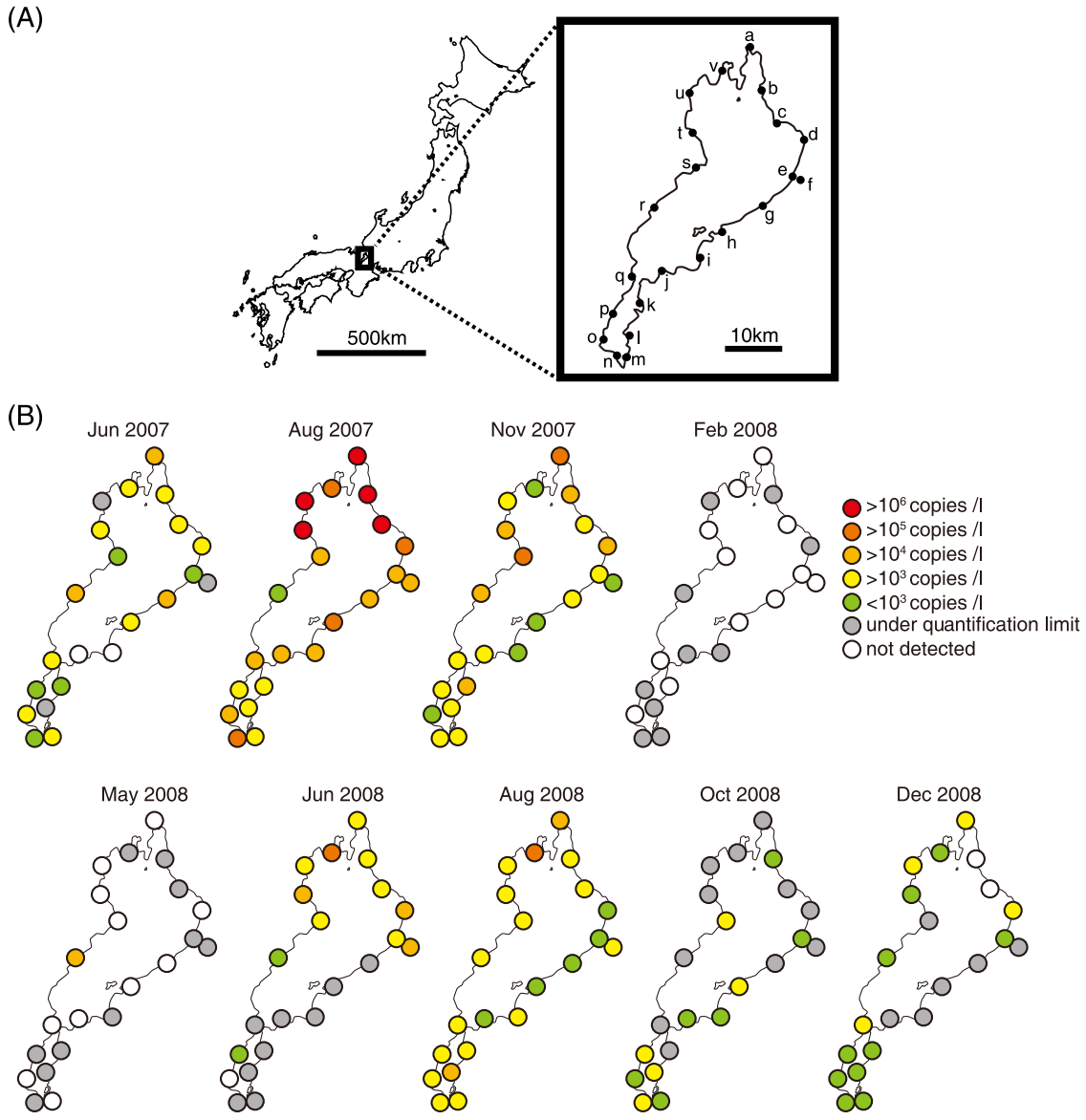

FIG. 1. (A) Study sites. The lake surface water was collected at 22 sites (sites a to v) on the coastline. The sampling sites are indicated by closed circles. (B) Seasonal distribution of CyHV-3 in Lake Biwa, Japan. Lake surface water was collected from 22 sites on nine occasions from June 2007 to December 2008. We calculated the CyHV-3 concentration (number of copies per liter) only when all three replicate samples showed positive results, and data were treated as "under the quantification limit" when only one or two positive results were obtained. Abbreviations: Jun, June; Aug, August; Nov, November; Feb, February; Oct, October; Dec, December.

lambda is 0.71 (Honjo et al., submitted), the mean CyHV-3 recovery yield was estimated as 8.9%, which was higher than that in a recent study (1.6%) in which the cation-coated filter method was adopted (7). Although our method is fundamentally the same as theirs, more elution fluid (4.4 times per area) was used in our study, which might be the reason for the higher recovery yields in the present study. Combining the estimated recovery yields and the quantification limit of real-time PCR (three copies per tube) (Honjo et al., submitted) revealed that the mean limit of our method was 170 copies liter^{-1}. Considering the results described below, our method is useful for quantifying CyHV-3 in the late spring, summer, and autumn when the CyHV-3 concentration is relatively high; however, further modification of the method with a higher recovery yield and/or greater water capacity is required in winter or early spring.

CyHV-3 distribution in Lake Biwa. CyHV-3 DNA was distributed all over the lake, even in winter (Fig. 1B). The mean concentration of CyHV-3 in water showed an annual oscillation with a peak in the summer and a trough in winter (Fig. 2). In Lake Biwa, relatively small numbers of dead carp infected with CyHV-3 have been found every year after the mass mortality in 2004 (Shiga Prefectural Fisheries Experiment Station, unpublished data). Such seasonal fluctuation in CyHV-3 concentration may have continued for years after the first outbreak in Lake Biwa. Most adult carp show a relatively high

TABLE 1. Characteristics of water samples from Lake Biwa, Japan

Sample name	Sampling dates	Mean (range) for characteristic:			
		Temp (°C)	Conductivity (mS/cm)	DO[a] concn (mg/liter)	pH
June 2007	15 May to 2 July 2007	25.1 (18.8–27.9)			7.2 (6.3–9.1)
August 2007	20 to 23 August 2007	30.8 (27.6–34.7)			7.7 (6.4–9.2)
November 2007	12 to 15 November 2007	16.4 (13.0–20.3)	0.17 (0.10–0.45)	9.5 (2.5–14.3)	7.8 (6.4–9.0)
February 2008	4 to 7 February 2008	8.2 (5.0–15.5)	0.13 (0.07–0.41)	12.2 (9.0–14.6)	7.4 (6.1–8.8)
May 2008	13 to 15 May 2008	18.7 (15.1–22.0)	0.14 (0.09–0.30)	10.8 (8.0–13.8)	8.1 (7.2–8.9)
June 2008	24 and 25 June 2008	23.9 (19.6–25.9)	0.16 (0.11–0.44)	9.3 (7.4–11.3)	8.5 (7.5–9.3)
August 2008	7 to 11 August 2008	32.0 (27.7–36.1)	0.20 (0.13–0.46)	9.6 (4.5–14.4)	8.7 (6.8–9.7)
October 2008	6 to 8 October 2008	22.2 (19.6–24.7)	0.16 (0.07–0.47)	9.3 (6.7–11.1)	8.0 (6.7–9.0)
December 2008	1 and 2 December 2008	13.1 (11.1–19.4)	0.14 (0.07–0.39)	10.1 (4.3–11.7)	7.6 (6.6–8.5)

[a] DO, dissolved oxygen.
[b] NTU, nephelometric turbidity units.
[c] VLP, virus-like particles.

CyHV-3 antibody level, which indicates past infection history (19). Generally herpesviruses that infect fishes show latent infection and reactivation (6, 14). Although CyHV-3 latency has not been confirmed, it is assumed that CyHV-3 also shows this feature (18). Accordingly, surviving fish become carriers that release the virus in the warm season and produce new infections and carriers. Thus, chronic presentation of CyHV-3 in Lake Biwa is conceivable. More generally, once CyHV-3 has invaded an aquatic environment, it will remain for a substantial period of time.

Most dead fish infected with CyHV-3 are found in late spring (June to July) and autumn (November to December) in Lake Biwa, Japan, and few are found in the summer. Since the temperature range of 18 to 25°C is required for disease development (15), such a pattern of infected fish detection is accepted as reasonable. However, the present study showed that the estimated CyHV-3 concentration is highest in August (Fig. 2). The reason for this discrepancy is unclear, but the activity of CyHV-3 might not be completely inert at such a high nonpermissive temperature (3). Further study is required to clarify this point.

A long-term question has been how CyHV-3 survives the winter season. Haramoto et al. (8) have reported the detection of CyHV-3 DNA from a river in winter. We also showed the presence of CyHV-3 DNA even in February when the water temperature falls below 10°C (Fig. 1B). These results suggest that the virus carriers release CyHV-3 even in winter when viral activity is predicted to be at its lowest. Given that carp and koi are the only natural hosts of the virus, CyHV-3 is predicted to retain its activity at nonpermissive low temperatures and the virus is released continuously from carp, even at a low level in the winter. Alternatively, CyHV-3 might survive the winter season in the lake sediment, and a small amount of virus may become detached from the sediment and appear in the surface water. This hypothesis is realistic, because active virus can be detected from carp droppings under experimental conditions (4). In this case, the bottom-feeding behavior of carp might be involved in spreading the virus/disease.

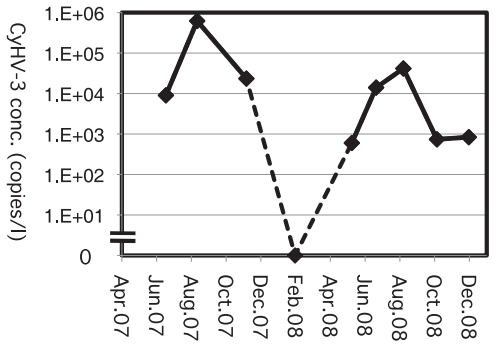

FIG. 2. Seasonal changes in mean CyHV-3 concentration. "Under quantification limit" and "not detected" were treated as 0. The mean concentration (number of copies per liter) of CyHV-3 in the water showed an annual oscillation, with a peak in August and a trough in February. Abbreviations: conc., concentration; Apr.07, April 2007; Jun.07, June 2007; Aug.07, August 2007; Oct.07, October 2007; Dec.07, December 2007; Feb.08, February 2008.

Correlation between CyHV-3 concentration and water quality. Although the detection of virus DNA does not directly mean the presence of active virus, understanding where the virus is present at high density is important to evaluate the risk of CyHV-3 disease as well as to clarify the ecology of the virus. We investigated the relationships between the CyHV-3 concentration and each water quality parameter obtained. The log CyHV-3 concentration showed a significant positive correlation with water temperature, turbidity, chlorophyll *a* concentration, and total bacterial count (Pearson correlation coefficients [r] = 0.31, 0.24, 0.26, and 0.19, respectively), and showed a significant negative correlation with the oxidation reduction potential (ORP) ($r = -0.39$) (Fig. 3) (see Table S3 in the supplemental material). Among these factors, the strongest correlation was that with the ORP. The result showed that more CyHV-3 particles are present at lower ORP sites, i.e., more reductive environments. This result supports the hypothesis that spawning sites are one source of infection (19), because the reed zones, the main spawning sites of carp in Lake Biwa, are generally muddy and reductive environments (9). In the highly turbid water, viruses may escape predation or degradation by attaching to organic or nonorganic particles. The chlorophyll concentration and the total bacterial count are indicators of eutrophication, and therefore, our results suggest that the CyHV-3 concentration increases in nutrient-rich sites. However, the mechanism involved remains unclear.

TABLE 1—*Continued*

Mean (range) for characteristic:						
ORP (mV)	Ammonia N concn (mg/liter)	Ammonium N concn (mg/liter)	Turbidity (NTU)[b]	Chlorophyll concn (μg/liter)	Total no. of VLP[c] (10^8 VLP/ml)	Total no. of bacteria (10^6 cells/ml)
					1.1 (0.4–4.7)	2.6 (1.5–4.7)
					0.8 (0.3–2.6)	4.5 (2.3–8.0)
214 (46–271)	0.01 (0.00–0.06)	0.34 (0.13–1.89)	19.0 (0.1–225.9)	8.2 (2.3–37.9)	1.0 (0.1–3.3)	4.9 (0.5–56.7)
313 (296–329)	0.00 (0.00–0.01)	0.14 (0.06–0.69)	3.9 (0.2–19.4)	6.1 (1.0–31.5)	0.6 (0.1–1.6)	1.2 (0.3–2.2)
259 (230–290)	0.01 (0.00–0.04)	0.21 (0.11–0.59)	14.6 (0.7–60.9)	11.2 (1.6–80.2)	0.7 (0.3–1.3)	3.2 (1.5–5.7)
259 (241–285)	0.08 (0.01–0.34)	0.35 (0.19–1.01)	9.6 (1.2–70.4)	8.2 (0.6–87.6)	0.8 (0.3–1.9)	3.5 (0.6–7.1)
221 (172–307)	0.54 (0.00–2.89)	0.47 (0.22–1.18)	2.0 (0.2–4.7)	4.5 (1.6–13.9)	1.0 (0.3–2.2)	3.7 (1.8–7.7)
300 (258–362)	0.02 (0.00–0.08)	0.28 (0.14–1.09)	5.1 (0.7–24.7)	5.9 (0.4–40.9)	0.9 (0.5–1.8)	3.0 (1.7–4.3)
355 (274–416)	0.01 (0.00–0.04)	0.61 (0.29–1.71)	9.9 (0.3–56.8)	2.9 (0.0–9.7)	0.7 (0.1–1.8)	1.8 (0.5–4.3)

Sequence comparisons with published genomes. Forty liters of lake surface water was collected at site q on 1 October 2008, and the viral DNA was collected and purified as described above. Genomic DNA fragments in which insertions/deletions (indels) and/or substitutions were observed among three published CyHV-3 genomes (1) were amplified with newly designed primer sets (Table 2), and amplified fragments were directly sequenced. The DDBJ accession numbers for these

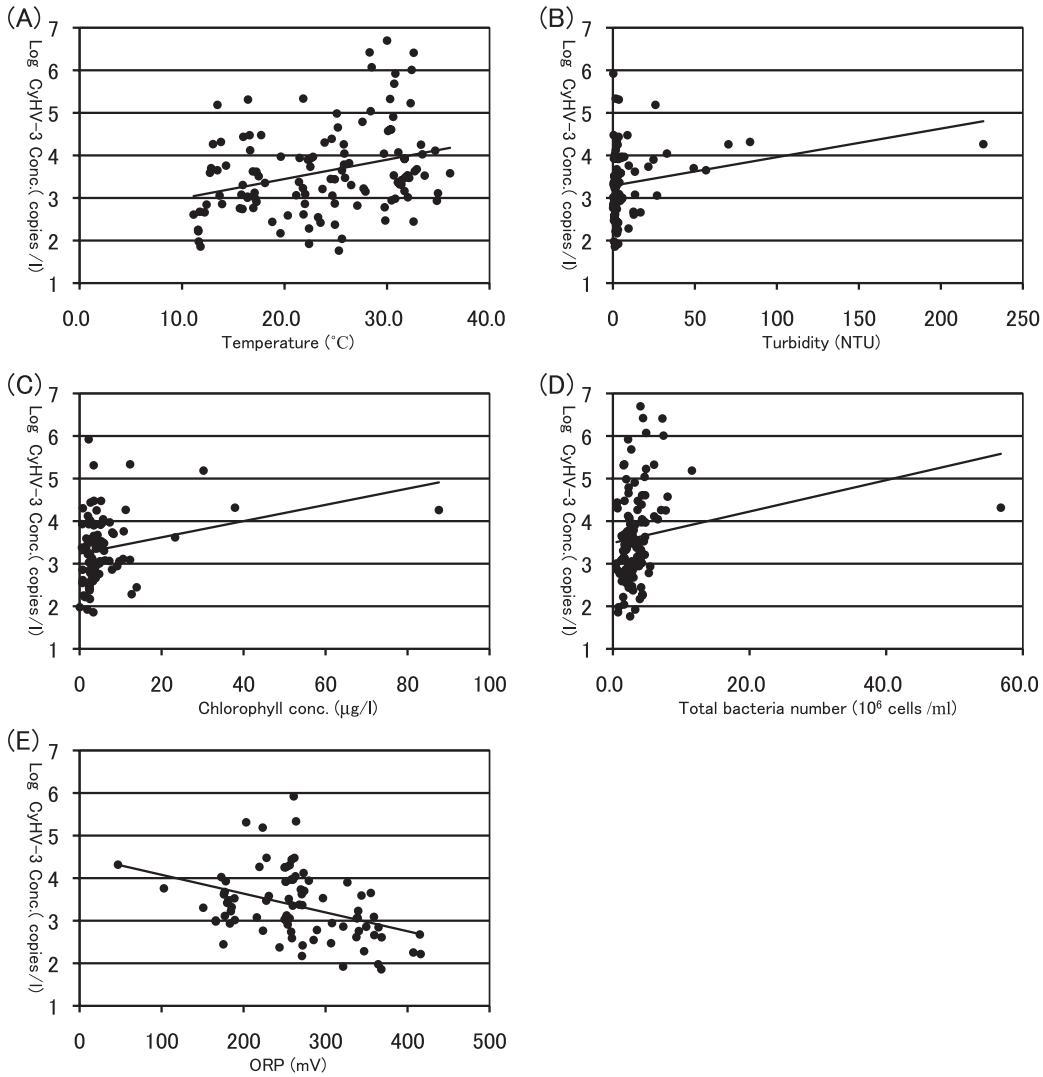

FIG. 3. Relationships between log CyHV-3 concentration and each water quality parameter. Only parameters with significant correlations are shown. (A to D) Water temperature, turbidity, chlorophyll concentration, and total numbers of bacterium-like particles showed a significant positive correlation with log CyHV-3 concentration (number of copies per liter) (r = 0.31, 0.24, 0.26, and 0.19, respectively). (E) ORP showed a significant negative correlation (r = −0.39). Regression lines are also shown. Abbreviations: Conc., concentration; NTU, nephelometric turbidity units.

TABLE 2. Detailed information on the PCR-amplified region for sequences

Target name	Target region positions (corresponding to GenBank accession no. AP008984)	Target length (bp)	Sequence of primer (5′→3′)		Annealing temp (°C)
			Forward primer	Reverse primer	
GV-1	32171–32611	441	ACAAACAGATTCAGGCACCC	TCGACTTTGACAAGCACCTG	63
GV-4	82976–83201	226	TCACGGACGTGATCAACAAT	CGGCAATGTAGGTTCTGGTT	60 or 63
GV-11	210559–211058	500	GTATAACAGCCGCCACGAAT	CGCTGGAACCTACACTGTGA	63
GV-12	212073–212808	736	CCGCGGACTGGTACTATCAT	GACCAAAGCTCCATGTCGAT	55 or 63
GV-13	221081–221477	397	GACCTTGGACATGTAGGCGT	CATGAGGCTACTCTGCCACA	55 or 60
GV-14	254153–254491	339	GTCAGCGTGCTTATGGGAAT	CTGGGAGTGTGCTGTCTTCA	55

fragments are AB505896 to AB505901. In an examination of the 2,399-bp sequence, the sequence of the Lake Biwa CyHV-3 genome matched completely that of the reported Japanese strain. Previously we sequenced CyHV-3 fragments obtained from Yura River, Japan, and the sequence also matched that of the Japanese strain (11). These results suggest that CyHV-3 obtained from Japanese environmental waters shares the same origins with the virus that caused mass mortality.

In this study, we demonstrated the seasonal distribution of CyHV-3 in Lake Biwa, Japan, though we cannot show the viral activity. To the best of our knowledge, this is the first report to show viral distribution patterns across seasons in the natural environment. Although the increasing/decreasing pattern did not coincide with the detection pattern of the infected fish, it gives new insight into our knowledge of CyHV-3 dynamics. Our results showed that CyHV-3 has remained for at least 5 years from the first outbreak, with seasonal fluctuation, which suggests the development of CyHV-3 in Lake Biwa. Understanding the ecology of CyHV-3 in natural environments may be useful in preventing the spread of CyHV-3.

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