Short communication

PCR-Detection of *Plecoglossus altivelis* Poxvirus-Like Virus (PaPV) in Wild Ayu

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ABSTRACT—*Plecoglossus altivelis* poxvirus-like virus (PaPV) is suspected as the causative agent for atypical cellular gill disease in ayu *P. altivelis*. The virus was detected by PCR from wild juvenile ayu and Japanese sardine *Sardinops melanostictus* caught in coastal areas, but not detected from the larval ayu caught in freshwater. No histopathological changes were observed in the gills of juvenile ayu positive for PaPV. When the positive juvenile population was reared in freshwater, the detection rate of the virus gradually decreased and reached below the detection limit 40 days after the stocking.

Key words: *Plecoglossus altivelis*, ayu, atypical cellular gill disease (ACGD), *Plecoglossus altivelis* poxvirus-like virus (PaPV)

Atypical cellular gill disease (ACGD) is considered to be related to the infection against *Plecoglossus altivelis* poxvirus-like virus, PaPV. The disease signs such as appetite reduction and poor swimming are usually observed in affected fish and mortality sometimes exceeds 90%. The previous histopathological studies revealed that PaPV-infected ayu showed proliferative branchitis with enlarged and atypical epithelial cells in the gills and that no pathological abnormality occurred in the other organs (Wada *et al.*, 2008; Wada *et al.*, 2011).

Most of the ayu populations are amphidromous, with a life span of only 1 year. The fish spawn in lower reaches of rivers in autumn and hatched larvae drift down into the sea. The juveniles spend about 5 months in the surf zone until they ascend rivers from spring to early summer (Senta *et al.*, 1985; Takahashi *et al.*, 1998). The fish grow and mature in the river. Most of the fish die after spawning.

There are three sources of ayu used for farming: wild amphidromous populations, the wild landlocked population caught in the Lake Biwa watershed, and juveniles reared in hatcheries from eggs. Fish are farmed in tanks or concrete ponds with flowing freshwater. Because the occurrences of ACGD have been reported in ayu farms, the presence of carrier organisms of PaPV in or around the farms has been suspected. Existence of such organisms, however, has never been reported so far, except for ayu. Interestingly, it is empirically known that ACGD occurs mostly among hatchery-produced fish. The disease has never been reported among cultured ayu raised from wild juveniles.

In this study, the wild larvae and juveniles of amphidromous ayu were tested for PaPV by the polymerase chain reaction (PCR) to identify whether they possess PaPV in nature. Additionally, other species were also tested for PaPV to know if there are hosts of the virus other than ayu.

Materials and Methods

Sampling of wild ayu, other fish and planktons

The larval ayu were caught in freshwater in December 2014. The early juveniles and juveniles were caught in seawater in December 2014 and in February 2015, respectively. All these samples were collected in Wakayama Prefecture and were immediately immersed in 99% ethanol. The other fishes caught in sea were also subjected to DNA preparation. Additionally, some live juvenile ayu were also transferred to Wakayama Prefectural Fishery Station. Some of them were sampled for histopathology and the others were reared in freshwater to monitor PaPV. Planktons were sampled from 1.0 m³ seawater by Kitahara quantitative plankton net (opening: ϕ 22.5 cm, mesh size: 72 μ m). The sample was centrifuged at 3,000 $\times$ g for 10 min, and was pelleted by centrifugation for DNA preparation.

DNA preparation

Total DNA of ayu, other fish and planktons were extracted using a DNA extraction kit (Sigma-Aldrich). Total DNA of larval ayu and plankton were extracted from whole bodies. Total DNA of the others were extracted from the gills. The numbers of fish used are summarized in Table 1.

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Table 1. Detection of PaPV by PCR in wild ayu and other fishes caught in river and coast

Sampling dates	Locations	Species	Average body weight	PCR Results ^{a)}
2014. Dec.	River A	<i>Plecoglossus altivelis</i> , larva	<1 mg	0/3* (30)
Dec.	Station B	<i>Plecoglossus altivelis</i> , early juvenile	14 mg	1/10* (10)
2015. Feb.	Station C	<i>Plocoglossus altivelis</i> , juvenile	0.83 g	3/3 (5)
Feb.	Station D	<i>Plocoglossus altivelis</i> , juvenile	0.39 g	5/5 (5)
Feb.	Station C	<i>Plocoglossus altivelis</i> , juvenile	0.58 g	6/6 (5)
Feb.	Station E	<i>Plocoglossus altivelis</i> , juvenile	0.51 g	3/3 (5)
Feb.	Station F	<i>Plocoglossus altivelis</i> , juvenile	0.53 g	3/3 (5)
Feb.	Station F	<i>Sardinops melanostictus</i>	0.80 g	1/3 (5)
Feb.	Station F	<i>Hypoatherina valenciennei</i>	1.78 g	0/2 (4)
Feb.	Station F	<i>Engraulis japonica</i>	1.38 g	0/1 (1)
Feb.	Station F	<i>Scomber australasicus</i>	0.80 g	0/1 (3)

The sampling point in River A and Station B locate at a distance about 3.0 km.

^{a)} Results of PCR are expressed as the number of positive samples per number of total samples tested. Asterisks mean that fish were alive when sampled. For the other cases, fresh dead fish were used. The numbers in parentheses indicate the numbers of pooled specimens per each sample.

PCR

DNA of PaPV was amplified from the template total DNA by the PCR^{*1,*2}. The primer set (BOKE30-F: cgatatcatatctgtgatcg and BOKE30-R: aatgttgatgtgtccag-gat) was used. The PCR mixtures (20 μ L) contained 2 μ L of 10 $\times$ PCR buffer, 2 μ L of 2 mM dNTPs, 1 μ L of each forward and reverse primer (1 pmol/ μ L), 0.5 μ L of ExTaq DNA polymerase (Takara Bio Inc.), 1 μ L of the template DNA (10 ng/ μ L), and 12.5 μ L of distilled water. The PCR was conducted under the following conditions: 95°C for 1 min for initial denaturation, followed by 30 cycles of amplification with heat denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec using Master Cycler Gradient (Eppendorf).

As a PCR positive control, the amplified PCR fragment of PaPV was acquired from Japan Fisheries Resource Conservation Association (JFRCA). The expected amplicon size of the PCR is 302 bp. The PCR products were analyzed in gel electrophoresis followed by ethidium bromide staining.

DNA sequencing

The PCR products of PaPV from the wild juvenile ayu and the sardine were sequenced by direct sequencing with the primer BOKE30-F or BOKE30-R. The sequences were aligned with the known PaPV partial sequence (AB747357) by Clustal X (Larkin *et al.*, 2007).

Histopathology

Live juvenile ayu ($n=5$, average body weight = 1.5 g) were subjected to histopathology, which were positive

in PaPV PCR. The gills of these fish were fixed in Davidson's solution and embedded in paraffin. The sections of 3–5 μ m were stained with haematoxylin and eosin (H&E).

Monitoring PaPV in juvenile ayu

A part of juvenile ayu (total 450 g, average body weight = 1.5 g) were reared in a 760-L tank filled with flowing well-freshwater at Wakayama Prefectural Fisheries Station. The fish were reared with feeding a commercial pellet food, Ayu Alpha Mega 2C (Nippon Formula Feed Manufacturing Company Limited) in the tank from 16 February to 30 April 2015. The water temperature was controlled at 14°C by a heater during the rearing period. The gills of each 30 fish were collected every 10 days from the start of rearing. The total DNA of the gills of each individual fish was extracted and tested for PaPV by PCR as described above.

Results

PCR-detection of PaPV

The detection of PaPV in the sampled fish is summarized in Table 1. PaPV were detected from the early juveniles and the juveniles of ayu caught in the surf zone at various locations. The virus was detected by a small percentage from the early juvenile that had spent a relatively short period of time (~1 month) in the sea. PaPV was also detected from a sample of juvenile sardine. In contrast, PaPV was not detected from the larval ayu and planktons.

Comparison of the partial DNA sequences of PaPV

The partial DNA sequence amplified from the wild juvenile ayu and from the sardine showed 100% identity with that of PaPV sequence deposited in GenBank (accession NO. AB747357).

*¹ Watanabe *et al.* (2007): Programs and abstracts, the Japanese Society of Fisheries Science, p. 221.

*² Japan fisheries resource conservation association (2011): Manual of diagnosis and treatment for atypical cellular gill disease in ayu *Plecoglossus altivelis*. (<http://www.fish-jfrca.jp/02/pdf/AYUACGD.pdf>), in Japanese.

Fig. 1. A histological section of the gills of a juvenile ayu specimen possessing PaPV. The section is stained with H&E. No pathological lesions including the formation of atypical cells, was observed.

Fig. 2. The detection rate of PaPV in amphidromous ayu caught in the sea and reared in a freshwater tank. For each data point, 30 fish were individually tested for PaPV by PCR. Closed circles indicate the detection rate of PaPV. Open squares indicate the water temperature.

Histopathology

No particular histopathological changes were observed in the gills, despite that PaPV was detected from these samples by PCR (Fig. 1).

Monitoring PaPV in juvenile ayu under freshwater rearing

The detection rate of the virus in captive juvenile ayu was 84% at the start of rearing (Fig. 2), at which appetite reduction and lethargic swimming were observed among the fish. The abnormal behaviors were recovered within 3 weeks. The detection rate of the virus was constantly decreased and no virus was detected in the ayu sampled 40 days after the stocking (Fig. 2). The cumulative mortality was less than 4.7% during the monitoring period. The ayu were continually reared for more than 6 months after the monitoring, but ACGD was not observed.

Discussion

PaPV had hitherto been considered to be distributed in freshwater, because the occurrence of ACGD has only been reported in ayu farms using freshwater. Nevertheless, no carrier organisms except for ayu had been identified in the surrounding area of ayu farms, and hence, the distribution of the virus was unclear. In contrast, the present study suggests that most of the wild ayu population were infected with PaPV in their early life stage while they were in the sea (Table 1). Interestingly, PaPV was also detected from the sardine that was caught along with juvenile ayu, but not from planktons. These results suggest that some of the natural hosts of the virus are juvenile ayu and sardine, which live in seawater. However, the specificity of PCR primers used in this study to detect PaPV has not systematically validated. Therefore, the virus related to PaPV might also have been detected by this PCR, although the PCR product was 100% identical to the known PaPV sequence. The future study using more fish samples would reveal the range of natural hosts of PaPV.

The virus detected from wild juvenile ayu seems not to be highly virulent, because the virus was cleared from the juveniles when they were reared in freshwater and the mortality of wild juveniles was very low during the observation. It is possible that PaPV is originally not so virulent to juvenile ayu, or the virus detected in the marine environment in this study is a less virulent strain of PaPV. The future comparative study including the genetics and the challenge test between the virus strains from marine juveniles and cultured ayu, will give a clue to reveal the pathogenic mechanism of the virus.

Notably, characteristic abnormalities of ACGD were not observed in the gills of juvenile ayu by histopathology (Fig. 1), despite that PaPV was detected in these specimens. The juvenile ayu reared in freshwater showed slight appetite reduction and lethargic swimming for 2 weeks from the start. Although it is uncertain whether these abnormalities were caused by PaPV

infection or the transfer from seawater to freshwater, the observation suggests that most of the wild juvenile ayu were in poor physical condition. There is a possibility that severely affected ayu had already died out and only surviving fish were caught and used in this study. The biology of larval ayu is only partially known and the causes of mortality of wild ayu population are not fully understood. The influence of PaPV against wild ayu stock should therefore be clarified.

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References

- Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins (2007): Clustal W and clustal X version 2.0. *Bioinformatics.*, **23**, 2947–2948.
- Senta, T. and I. Kinoshita (1985): Larval and juvenile fishes occurring in surf zones of western Japan. *Trans. Am. Fish. Soc.*, **114**, 609–618.
- Takahashi, I., K. Azuma, S. Fujita and I. Kinoshita (1998): Spatial distribution of larval ayu *Plecoglossus altivelis* in the Shimanto Estuary, Japan. *Fish. Sci.*, **64**, 522–525.
- Wada, S., O. Kurata, K. Hatai, H. Ishii, K. Kasuya and Y. Watanabe (2008): Proliferative branchitis associated with pathognomonic, atypical gill epithelial cells in cultured ayu *Plecoglossus altivelis*. *Fish Pathol.*, **43**, 89–91.
- Wada, S., H. Atami, O. Kurata, K. Hatai, K. Kasuya, Y. Watanabe and H. Fukuda (2011): Histopathology of gill lesions of ayu *Plecoglossus altivelis*. clinically diagnosed with 'Boke' disease. *Fish Pathol.*, **46**, 59–61.