Special Issue

Development of DNA diagnostic methods for the detection of new fish iridoviral diseases

Tadakazu Tamai¹, Kazunari Tsujimura², Sanetaka Shirahata², Hiroshi Oda¹, Tohru Noguchi², Riichi Kusuda³, Nobuyuki Sato¹, Shoji Kimura¹, Yoshinori Katakura² & Hiroki Murakami^{2†*}
¹ Maruha Central R&D Institute, Maruha Co., Ltd., 16–2 Wadai, Tsukuba-shi, Ibaraki 300–12, Japan; ² Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka 812–81, Japan; ³ Faculty of Agriculture, Kouchi University, 200 Mononobe Otsu, Nangoku-city 783, Japan

Received 23 July 1996

Key words: fish iridovirus, PCR diagnosis, hybridization assay

Abstract

A new disease of epidemic proportions caused by fish viruses within the *Iridoviridae* family inflicts serious damage on red sea breams (*Pagrus major*) and striped jack (*Caranx delicatissimus*) populations grown in aquacultures in Japan. A partial segment of the fish iridoviral DNA was directly amplified using the polymerase chain reaction (PCR) with synthetic primers designed from well conserved nucleotide sequences between the frog virus 3 (*Ranavirus*) and the silkworm iridescent virus type 6. The deduced amino acid sequence from the nucleotide sequence of the PCR fragment demonstrates a high correlation with a partial sequence from the frog virus 3. Using the PCR method with specific primers, we could detect three of four different known types of fish iridoviruses in diseased fishes. To construct more reliable detection methods specific for this viral family, DNA fragments which can specifically hybridize with all of the four known *iridoviridae* viral DNAs were screened from the genomic library of one *iridoviridae* strain. The hybridization assay, using a specific fragment which contains regions which are highly homologous with a characterized partial sequence from the frog virus 3, proved to be a reliable diagnostic tool for fish iridoviral diseases.

Introduction

A new epizootic disease which causes severe anemia resulting in high mortality has been reported in aquacultures of fishes such as red sea bream (*Pagrus major*), striped jack (*Caranx delicatissimus*) and yellow tail in Japan since 1990. From pathological and virological studies, these epizootic diseases were demonstrated to be caused by new types of fish virus from the *Iridoviridae* family (Inouye *et al.*, 1992). Hexagonal virions without an envelope (200–240 nm in a diameter) were detected in the cytoplasm of leukocytes found in dis-

eased fishes. In an afflicted fish, these viruses cause Irido symptoms such as bleeding on the epidermal surface, cell hypertrophy in spleen, and finally death. Because the infectiousness of the virus is weak, thereby being difficult to subculture, the characterization of these viruses have not been done thoroughly.

Conventional diagnosis of fish viral diseases is based upon appearance of lesions, histological analysis of hypertrophic cells, electron microscopic detection of viral virions in tissues, and serological or histological analysis using antibodies. Since these diagnostic procedures are often not very sensitive, we investigated ways diagnostic procedures can be made more rapid, sensitive, and easier. For this purpose, we have

* † The late Professor. This paper was devoted to the soul of the late Professor Hiroki Murakami.

established murine monoclonal antibodies against fish iridovirus (*Iridoviridae*) (Oda *et al.*, 1993).

Polymerase chain reaction (PCR) is an extremely sensitive assay that is widely used for the diagnosis of viral diseases and the identification of viruses (Delaporte *et al.*, 1991; Escaich *et al.*, 1991; Rodriguez *et al.*, 1992). For efficient PCR amplification, the design of the primers, taken from homologous regions of known sequences from various viruses within the same family, is essential. Since viruses are often associated with mutational changes, it is desirable to select a region where the degree of intertypic homology is higher. However, none of the fish *Iridoviridae* viral DNA sequences are known and only two partial sequences from *iridoviridae* viruses, one infectious to frogs and the other to silkworm, were available in the EMBL/GenBank database.

Other diagnostic methods use different approaches to detect viral DNAs. The DNA hybridization technique uses DNA probes containing consensus sequences specific to all members in a certain viral family to detect specific viral sequences. Although viral DNAs are inherently variable, viruses which belong to the same family usually have highly conserved, stable partial sequences. Here we report that the PCR primers designed from a limited amounts of genetic information and a DNA probe screened from the genomic library of a particular *iridoviridae* strain are applicable for the PCR and DNA diagnosis of the fish iridoviral diseases.

Materials and methods

Preparation of viral DNA template for PCR reaction

The fish virus, *Iridoviridae* (TI92 strain) was isolated from the spleen of diseased fishes (Oda *et al.*, 1993). The TI92 virus was multiplied in the PL-51 cell line, which is an established flatfish lymphocyte cell line immortalized with both the human c-Ha-ras and human c-fos oncogenes (Tamai *et al.*, 1993). PL-51 cells (8×10^5 cells/ml) were challenged with TI92 in ERDF medium (Kyokuto Seiyaku, Tokyo) supplemented with 5% fetal calf serum (FCS) (Whittaker, USA). After incubation at 15 °C in an atmosphere of 5% CO₂-95% air for 7 days, the cultured medium was stocked for future use as a crude TI92 solution. Other types of *iridoviridae* virus, MI-5, MI-10 and SI-1, were isolated from diseased red sea breams and striped jack, and were multiplied in BF-2 cells

which are derived from Blue gill. Infectious pancreatic necrosis virus (IPNV, VR299 strain) (Kamei *et al.*, 1987), infectious haematopoietic necrosis virus (IHNV, ChAb strain) (Kamei *et al.*, 1987), Hiram rhabdovirus (HRV: *Rhabdovirus olivaceus*, 840-H strain) and oncorhynchus masou virus (OMV) (Kamei *et al.*, 1987) were propagated in CHSE (Chinok Salmon Embryo) cells (Kamei *et al.*, 1987). These viruses were purified from crude preparation using the method developed by Chang *et al.* (1978). To isolate viral DNA, purified virus particles were mixed with 200 µg/ml proteinase K and 2% sodium dodecyl sulfate (SDS), then incubated at 65 °C for 1 hr, followed by DNA extraction with phenol/chloroform.

Oligonucleotide primer design

As shown in Figure 1A, from partial sequences in the genomes of frog virus 3 (*Ranavirus*) (Willis *et al.*, 1984) and insect iridescentvirus 6 (Fischer *et al.*, 1988) which were found in the EMBL/GenBank database, seven highly homologous regions were shown to be conserved in both virus types. The PCR primers designed from these consensus sequences were synthesized using a Cyclon Plus DNA synthesizer (Milli-Gen/Biosearch, USA). The sequences are as follows: Primer 1, 5'-TAGATTTGGTGAACCTGTCTG-3'; primer 2, 5'-AACCTTTGTGGTGGAGCCCC-3'; primer 3, 5'-CATTCGACGGGATTGAGCC-3'. A pair of inner primer, primer 4 (5'-GACTAATAACAATGCGGTTA-3') and 5 (5'-AGGCTGCTGCTTTCCCTCTG-3'), were designed based on fish iridoviral DNA sequences (Figure 2). All primers were dissolved in TE buffer (pH 8.0).

Polymerase chain reaction

The PCR reaction mixture (20 µl) contained 1 x PCR buffer (50 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂ and 0.01% gelatin), 200 mM dNTP and 0.25 unit of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk), 1 mM of each primer and template DNA. The mixture containing primers 1, 2 and 3 was amplified under the following conditions: 35 successive cycles of heating at 94 °C for 0.5 min, renaturing at 37 °C for 10 min, and polymerization at 55 °C for 7 min. Likewise, the following conditions, 35 successive cycles of heating at 94 °C for 1 min, renaturing at 52 °C for 2 min, and polymerization at 70 °C for 1 min, was used for amplifying the mixture containing primers 4 and 5.

Figure 1. Homology between the partial genomes of *Ranavirus* and iridescent virus, and the specific primers for PCR amplification. (A) a nucleotide sequence found in the immediate early frog virus 3 gene with the four open reading frames which encodes the iridescent virus type 6 are shown with the conserved segments. The three consensus segments that were used to design the PCR primers for amplification of the fish iridovirus genome are shown below. (B) PCR amplification of the fish TI92 *iridoviridae* genome using the mixture of primer 1, 2, and 3 were run under the following conditions; melting 94 °C, 1 min; primer annealing 37 °C, 2 min; primer extension 65 °C, 5 min; cycle number, 35.

The PCR products were detected by electrophoresis on a 1% agarose gel.

DNA sequencing

A fish iridoviral PCR fragment of reasonable size (ca. 200 bp) was purified by 1% agarose gel electrophoresis and ligated into the pUC119 sequencing vector. Dideoxynucleotide chain termination sequencing was carried out using modified T7 DNA polymerase (Sequenase) (Biochemical Corp., Cleveland, OH). To eliminate the PCR-induced error, four independent clones were sequenced.

Statistical analysis of sequence homology

Homology between two sequences (a and b) was expressed as a Z value (Barker and Dayhoff, 1972): $Z = (R - M) / SD$, where R is the score for the best alignment between a and b, M is the mean value of best alignment scores for 50 pairs of randomly arranged sequences, each having the same base composition as a or b, and SD is the standard deviation of those 50 scores. Penalty was 5. Scoring of the best alignment was made by the maximum matching method (Needleman and Wunsch, 1970), assigning +1 for each match, 0 for each mismatch, and -2 for a gap. The probability of

attaining the homology level shown between sequences a and b by random arrangement of nucleotides is related to the Z value: 0.001 ($Z=3.73$), 0.0010 ($Z=3.09$), 0.0100 ($Z=2.32$), 0.1000 ($Z=1.28$), and so on.

Specificity assay of the PCR reaction

Six murine monoclonal antibodies (SM-803, -804, -805, -807, -808, and -809) specific to fish iridovirus (*Iridoviridae*) family were generated as described previously (Oda *et al.*, 1993). All strains of iridovirus (TI92, MI-5, MI-10 and SI-1) could be detected by the enzyme-linked immunosorbent assay (ELISA) (Eto *et al.*, 1991) using these monoclonal antibodies.

IPNV, IHNV, OMV and HRV were propagated in CHSE cells or EPC cells and the virus virion prepared as described before were lysed by boiling for 5 min to isolate the viral DNA templates. The PCR amplification was done using these viral DNA templates under the same conditions as described above.

Spleens and brains were collected from diseased striped jack and red sea bream showing the irido-symptom. After microscopically observing cell hypertrophy, they were disrupted with a glass homogenizer. The homogenates were centrifuged at 15,000 x g for 10 min to remove debris, and the supernatant were filtered through a 0.45 μ m membrane. These filtrates

(A)

(B)

Figure 2. Homology comparison of partial nucleotide and amino acid sequences from the fish *Iridoviridae* genome and the frog iridovirus. (A) comparison of nucleotide sequences from the fish iridovirus PCR fragment (upper lane) and frog iridovirus (lower lane). The conserved parts are denoted with asterisks and the primer segment are boxed. (B) The upper lane shows the deduced partial amino acid sequence from the fish iridovirus. The lower lane shows the sequence of the segment from the frog virus 3. The iridovirus sequence was aligned with gaps to increase similarity with that of the frog virus 3. Sequences corresponding to the primer segment were boxed. One asterisk shows a similar, molecular evolutionarily, amino acid. Two asterisks show an identical amino acid.

were used as a crude virus preparation and stored frozen until needed for use. Controls (virus-free tissue extracts) were prepared from healthy striped jack and red sea bream showing none of the irido-symptom.

Colony hybridization

Viral DNA derived from the *iridoviridae* strain MI-10 was cleaved with either Not I or PvuII and ligated into pBluescript to construct a genomic DNA library. Colonies were grown on an antibiotic containing agar

Figure 3. Specificity of the PCR reaction. (A) PCR amplification of a 200 bp specific fragment from the spleen crude extract of diseased striped jack infected by TI92 iridovirus. Healthy fish showing none of the irido-symptom was used as control. (B) Iridovirus specific PCR amplification. Various fish virus DNAs derived from IPNV, IHNV, HRV and fish iridovirus TI92 were used as templates for PCR amplification. The PCR conditions are the same as described in Figure 1, with exceptions that primer 4 and 5 were used instead of primer 1, 2, and 3 and the annealing temperature was 50 °C.

plate. A nylon membrane was then placed on the master agar plate. Replica membranes were prepared by membrane-membrane contact. Colony hybridization was done using the method of Sambrook *et al.* (1989). The DNA probes were labelled using the Megaprime DNA Labeling system (Amersham). One ng of ^{32}P -labelled SI-1 DNA (1×10^6 cpm) was added into the hybridization bag to hybridize with genomic DNAs fixed to the membrane, and was incubated overnight. The membranes were exposed to an X-ray film and positive colonies were then picked up from replica agar plate.

Dotblot hybridization

The MI-10 iridoviral DNA was digested with PvuII or NotI and labelled with α - ^{32}P -dCTP (3,000 Ci/mmol) using a random primer labelling kit (Amersham) to produce a hybridization probe. Ten μg of SI-1, MI-5 and MI-10 viral strain DNAs were blotted onto a nylon membrane (Hybond-N⁺; Amersham), and hybridized with 1 ng of the digested and labelled MI-10 DNAs (1×10^9 cpm/mg) at 60 °C for overnight. The membrane was washed at 60 °C in 2 x SSC-0.1% SDS twice for 15 min each and twice in 0.1 x SSC-0.1% SDS for 15 min each, then exposed to an X-ray film. Ten μg of pBluescript DNA was used as a control.

Results

Design of the PCR primers for amplification of the fish virus Iridoviridae genomes

Only two viral genomes belonging to the *Iridoviridae* family were found in the EMBL/GenBank database. One is from the frog virus 3 gene which belongs to the *Ranavirus* family. The other is from the insect iridescentvirus type 6 which infects silkworms. A homology search of the two genomes reveals that there are seven regions that are more than 70% homologous with each other (Figure 1A), although the whole known genomic homology shared between the two viruses is low. From these the high homology regions, three regions were chosen to design PCR primers (primers 1, 2 and 3) which were able to amplify fragments of about 200 bp long. PCR amplification of the TI92 virus genome using a mixture of primers (1, 2 and 3) under unstringent conditions demonstrated a smear DNA band containing a faint 200 bp band (Figure 1B). This 200 bp band was not observed either when a higher annealing temperature than 37 °C was used, or when extracts of healthy red sea breams were used as target.

Figure 4. PCR diagnosis of fishes infected with the iridoviruses. DNA templates were prepared from the spleen or the brain of various kinds of infected fish. The PCR conditions are the same as described in Figure 3. Lane 1, spleen of SI-1-infected striped jack; lane 2, spleen of MI-10-infected sea bream; lane 3, spleen of MI-5-infected sea bream; lane 4, spleen of TI92-infected striped jack; lane 5, brain extract of TI92-infected striped jack; lane 6, control using cultured medium of PL51 cells.

Sequence analysis of the PCR-amplified fish Iridoviridae genome

The 200 bp PCR fragment was subcloned into the pUC 119 vector and sequenced. The nucleotide sequence of the cloned PCR fragment except for the primer sequences shares about 67% identity with that of the frog virus 3 (EMBL/GenBank accession No.: U15575) without counting the gaps (Figure 2A). The amino acid sequence predicted from the nucleotide sequence was aligned with two amino acid gaps to increase similarity with the frog virus 3 sequence. As shown in Figure 2B, the predicted amino acid sequence of the fish iridoviral PCR fragment is only 13% identical with the partial sequence obtained from frog virus 3 except for the sequences corresponding to the primer segments, without counting the gaps. However, when taking evolutionary mutation into account, amino acid similarity between the PCR fragment and frog virus 3 is 59%, suggesting that these genes are related to each other. No significant similarity between the amino

acid sequences of the PCR fragment and insect iridescentvirus type 6 was found (data not shown).

Specificity of the PCR method

In order to achieve more specific amplification, a pair of primers (primer 4 and 5) were synthesized based on the inner sequences found in primer 1 and 3 in the 200 bp PCR fragment. Primers 4 and 5 specifically amplified the 200 bp band even at a high annealing temperature of 50 °C. A strong 200 bp band was detected from diseased fish sample (Figure 3A). Although a faint band was also detected in PCR reaction using a healthy fish sample, we cannot assure that there was no viral infection in healthy fish in appearance. The band was amplified only from the iridovirus genome, not from other fish viruses genomes such as IPNV, IHNV, HRV or OMV (Figure 3B). These results suggest that the PCR amplification reaction is specific to the fish virus of the *iridoviridae* family.

PCR diagnosis of fishes infected with the Iridoviridae viruses

To determine whether use of the PCR assay is possible for diagnosing of virus infected fish, the spleen or brain extracts of various fish infected with a different strain of the *Iridoviridae* family were used as PCR samples. The primer pair (primer 4 and 5) detected the single 200 bp DNA fragment from tissue extracts of fishes infected with the TI92, MI-5 and MI-10 strains, but not with the SI-1 strain (Figure 4). These 4 strains of iridovirus (*Iridoviridae*) were recognized by murine monoclonal antibodies. The PCR amplification method also could not detect purified SI-1 virus, which originated from an infected striped jack and was propagated in BF-2 cells (data not shown). The inability to detect the 200 bp fragment in the SI-1 strain may be due to a wide viral mutation located near the primer regions. Even when using primers with sequences in a slightly different frame from primer 1, 2, 4, and 5, we could not detect the 200 bp fragment. Six different kinds of monoclonal antibodies specific to fish iridovirus reacted with all of the four types of viruses, TI92, MI-5, MI-10, and SI-1, suggesting that SI-1 virus still belongs to the *Iridoviridae* family (data not shown).

Table 1. Reactivities of Fragment 12, 12-a, and 12-b to various fish iridoviral DNAs.^a

Probes	Fish iridoviral DNAs				
	Control	TI92	SI-1	MI-5	MI-10
Fragment 12	–	+	+	+	+
Fragment 12-a	–	+	+	+	+
Fragment 12-b	–	–	+	–	+

^a Fragment 12 was digested with *Pst* I to produce Fragment 12-a and 12-b. The fragments were labelled and hybridized with various fish iridoviral DNAs as described in Figure 6. Control, pBluescript vector DNA; +, positive; –, negative.

Cloning of the DNA fragment containing the fish iridovirus specific consensus sequence

Because the 200 bp PCR product could only be detected in 3 of 4 iridoviral genome DNAs, we attempted to find a DNA fragment that would be able to hybridize to all four genomic DNAs. First we digested the entire genome of strain MI-10 with *Pvu*II or *Not* I separately, then inserted the fragments into the pBluescript vector to construct a genomic DNA library. Dot blot hybridization demonstrated the existence of DNA fragments in the MI-10 genomic library which was able to hybridize to parts on all the other fish iridoviral DNAs (Figure 5). Positive clones were first screened from the MI-10 genomic library first using the genomic DNA from SI-1 as a probe, then hybridized with DNAs from the remaining strains using the dotblot hybridization method. Fragment 12 was found to be able to hybridize to all 4 iridoviral strains (Figure 6B). The size of this fragment is about 0.9 kbp and contains the *Hind*III, *Spe*I and *Pst*I restriction sites (Figure 6A). Fragment 12 was digested with *Pst*I and the resulting 2 fragments were named Fragment 12-a and 12-b. These fragments were able to hybridize with iridoviral DNAs by themselves. Fragment 12-b was able to hybridize with two of the four fish iridovirus DNAs, while Fragment 12-a had the ability to hybridize with all of the fish iridovirus DNAs (Table I). These results suggest that Fragment 12-a contains a fish iridovirus specific consensus sequence.

DNA analysis of Fragment 12-a and the nucleotide sequence comparison between the fish iridovirus and frog virus 3

The determined nucleotide sequence for Fragment 12-a is shown in Figure 7. This fragment is 465 bp in length and has a base composition of 43.4% G+C.

The nucleotide sequence for Fragment 12-a has about 47% identity with that encoding a mature peptide of frog virus 3 (EMBL/GenBank accession No.: M19872. Beckman *et al.*, 1988). Noteworthy are the 35 bp highly homologous region from the nucleotide position 143 to 177 in Fragment 12-a, which share 83% identity with that of frog virus 3. Homology of amino acid sequences of Fragment 12-a and frog virus 3 was analyzed by the computer program “ALIGN” with Dayhoff’s mutation matrix (250 PAMs) to give a Z value (high values indicate higher homology) for both fragments. A relatively high Z value of 4.64 was obtained for Fragment 12-a and frog virus 3, indicating that the Fragment 12-a sequence is related to the frog virus 3 partial sequence.

Discussion

PCR has been applied as an extremely sensitive and rapid diagnostic procedure for detecting viral diseases, but is limited by the requirement for specific primers. Since virus genomes are easily changeable, it is desirable to compare as many viral sequences of the same species as possible to search for highly conserved regions (Rodriguez *et al.*, 1992). So far, PCR diagnosis for fish viruses has not been done widely, perhaps due to poor information concerning the primary structures of fish viruses. Here, we have demonstrated that even with only minimum information about iridoviruses, we were successful in detecting fish iridoviruses with the PCR. From this we were able to determine the partial sequence of the fish iridovirus. The predicted amino acid sequence of the PCR fragment showed a high similarity with frog virus 3, but not with insect iridescent virus type 6.

Using the specific PCR primers, the 200 bp DNA fragment could be detected in 3 fish iridovirus strains (TI92, MI-5 and MI-10) but not in the SI-1 strain. Since all the four strains are recognizable by monoclonal antibodies specific to the fish iridoviruses, SI-1 may have wide ranging viral mutations. This fact demonstrates that PCR diagnosis can distinguish differences between various members of the same family, whereas these differences can not be recognized by monoclonal antibodies. However, the presence of a strain which can not be detected by PCR also demonstrates the limitation of the PCR diagnosis.

DNA hybridization assay is also used to detect viral specific consensus sequences. We produced Fragment 12-a by generating a genomic library of a particular iridovirus strain. This fragment was proven to possess an

Figure 5. Dotblot hybridization of fish iridovirus DNAs with various viral DNA probes. Experimental conditions were described in "Materials and methods". Probes: A, the PCR amplified 200 bp fragment ; B, Puv II digested MI-10 DNA ; C, Not I digested MI-10 DNA.

Figure 6. Restriction map of Fragment 12 (A) and a dotblot hybridization of Fragment 12 with various fish iridovirus DNAs (B). The NotI digested MI-10 DNA was inserted into the multicloning site of pBluescript to construct a genomic DNA library. Fragment 12 was obtained from a positive clone which hybridized with SI-1 DNA from the genomic DNA library. (A) restriction map of Fragment 12. (B) dotblot hybridization analysis.

iridovirus specific consensus sequence. The hybridization technique using the Fragment 12-a made it possible to detect all of four fish iridoviruses examined. The partial sequence, from 143 to 177, in Fragment 12-a exhibited a homology as high as 83% with the frog virus 3 sequence. This sequence will be useful in designing more reliable primers for the PCR diag-

nosis of fish iridovirus as well as to obtain a more specific DNA probe. The fact that both the PCR fragment and the Fragment 12-a exhibited higher similarity with frog virus 3 than with insect iridescentvirus type 6 suggests that the fish iridoviruses may belong to the genus *Ranavirus* of the family *Iridoviridae*. Various fish iridoviral diseases have been reported so

- Oda H, Tamai T, Tsuzimura K, Sato N, Kimura S, Shirahata S & Murakami H (1993) Establishment and characterization of monoclonal antibodies against fish virus (*Iridoviridae*). In: Kamino-gawa S *et al.* (eds), *Animal Cell Technology: Basic & Applied Aspects*, Vol 5, pp 565–571, Kluwer Academic Publishers, Dordrecht.
- Reno P W & Nicholson BLJ (1981) Ultrastructure and prevalence of viral erythrocytic necrosis (VEN) virus in Atlantic cod, *Gadus morhua* L., from the northern Atlantic Ocean. *J Fish Dis* 4: 361–370.
- Rodriguez A, Martinez-Salas E, Dopazo J, Davila M, Saiz JC & Sobrino F (1992) Primer design for specific diagnosis by PCR of highly variable RNA viruses: typing of foot-and-mouth disease virus. *Virology* 189: 363–367.
- Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning. A laboratory manual* 2nd Edition. Cold Spring Harbor Laboratory Press.
- Shchelkunov IS & Shchelkunova TI (1984) Results of virological studies on gill necrosis. *Symposia Biologica Hungarica* 23: 31–43.
- Smail DA & Egglestone SI (1980) Virus infections of marine fish erythrocytes: electron microscopical studies of the blenny virus. *J Fish Dis* 3: 45–47.
- Tamai T, Shirahata S, Sato N, Kimura S, Nonaka M & Murakami H (1993b) Purification and characterization of interferon-like antiviral protein derived from flatfish (*Paralichthys olivaceus*) lymphocytes immortalized by oncogenes. *Cytotechnology* 11: 121–131.
- Willis D, Foglesong D & Granoff A (1984) Nucleotide sequence of an immediate-early frog virus 3 gene. *J Virology* 53: 905–912.
- Wolf KMG & Malsberger RG (1966) Lymphocytis virus; isolation and propagation in centrarchid fish cell lines. *Science* 151: 1004–1005.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence database with the following accession number D38518 and D38519.

Address for correspondence: Sanetaka Shirahata, Ph.D. Graduate School of Genetic Resources Technology, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan.
Tel: +81–92–642–3045, Fax: +81–92–642–3052