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Differentiation of Lymphocystis Disease Virus Genotype by Multiplex PCR

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Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease. The viruses have been divided into three genotypes (genotype I for LCDV-1, II for Japanese flounder isolates, and III for rockfish isolates) on the basis of major capsid protein (MCP) gene sequences. In this study, we developed a multiplex PCR primer set in order to distinguish these genotypes. We also analyzed the MCP gene of a new LCDV isolate from the sea bass (SB98Yosu). Comparison of sequence identities between SB98Yosu and eight Japanese flounder isolates, revealed identity of more than 90.1% at nucleotide level and 96.5% at deduced amino acid level, respectively. Phylogenetic analyses based on the MCP gene showed that SB98Yosu belongs to genotype II, along with Japanese flounder isolates. Multiplex PCR based on the MCP gene allowed us to identify these genotypes in a simple and rapid manner, even in a sample that contained two genotypes, in this case genotypes II and III.

Keywords: lymphocystis disease virus (LCDV), multiplex PCR, genotype

Lymphocystis is a chronic, benign viral disease, which affects approximately 100 species of marine and freshwater fishes (Wolf, 1988). Fishes affected with lymphocystis disease (LCD) exhibit a characteristic external symptomology with clusters consisting of enormously hypertrophied dermal cells on the skin and fins. The hypertrophic cells, generally referred to as lymphocystis cells, possess a thick hyaline capsule, an enlarged nucleus, and prominent basophilic DNA cytoplasmic inclusions (Peters and Schmidt, 1995). In Korea, LCD is a common fish disease, and mainly affects Japanese flounder, *Paralichthys olivaceus*, rockfish *Sebastes schlegeli* and sea bass, *Lateolabrax* sp. Although LCD is associated with a relatively low mortality rate, the disfigurement it induces in infected fish generally renders them unsalable.

Lymphocystis disease virus (LCDV), the causative agent of LCD, belongs to the genus *Lymphocystivirus*, within the family *Iridoviridae*, and possesses an icosahedral capsid of approximately 200±50 nm in diameter. The LCDV capsid is double-layered, with an outer envelope and a fringe consisting of fibril-like external protrusions (Walker, 1962; Zwillenberg and Wolf, 1968; Wolf, 1988). The viral genome is a single linear dsDNA molecule, the structure of which is circularly permuted and terminally redundant (Darai *et al.*, 1983; Schnitzler *et al.*, 1987; Schnitzler and Darai, 1993). The complete genome sequences of two LCDV strains, LCDV-1 from flounder *Platichtys flesus L.* in Europe and LCDV-C from Japanese flounder in China, were determined. The former is 102,653 bp in length and encodes for 195 potential ORFs, whereas the latter is 186,250 bp in length, with 240 potential ORFs (Tidona and Darai, 1997; Zhang *et al.*, 2004). Upon comparison of the LCDV-1 and LCDV-C genomes, the highest degree of nucleotide sequence identity (78.9%) was observed in a major capsid protein (MCP) gene, which encodes for a single polypeptide with a molecular mass of 50 kDa (Schnitzler and Darai, 1993; Tidona and Darai, 1997; Zhang *et al.*, 2004). The MCP gene is one of the most important with regard to analyses of genetic relationships among iridoviruses, as it is relatively conserved among viruses in this family (Tidona *et al.*, 1998; Chinchar *et al.*, 2005). In addition, we recently reported that at least three genotypes were present in the genus *Lymphocystivirus*, on the basis of MCP gene sequence and the pathogenicities of lymphocystiviruses; i.e., genotype I includes only LCDV-1; genotype II consists of Japanese flounder isolates; and the remaining genotype, genotype III, consists of rockfish isolates (Kitamura *et al.*, 2006).

Although LCDV has been previously well-characterized both from a pathological and molecular standpoint, little

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remains regarding the manner in which the virus is spread, or how it might be detected. The most frequently-utilized technique for the detection of viruses is virus isolation and neutralization using cell lines. However, very few cell lines have proven to be susceptible to LCDV (Perez-Prieto et al., 1999, Iwamoto *et al.*, 2002). García-Rosado *et al.*, (2002) previously reported that serological techniques, including indirect immunofluorescence and flow cytometry using an anti-LCDV polyclonal antibody, could be useful for the virus detection in infected cells, but they noted the necessity for molecular techniques for the rapid detection of LCDV. In this study, therefore, a multiplex PCR based on MCP gene sequences was developed, in order to enable the fast and precise detection and typing of lymphocystiviruses. We also analyzed the MCP gene of a new LCDV isolate from sea bass in Korea.

Ten LCDV isolates reported previously by Kitamura *et al.*, (2006) (JF00Yosu, JF03GunNeA, JF03GunNeB, JF03ShinJi, JF04Jeju, JF00Kuma, JF03Yoshi, RF03Yosu, RF04Yosu and RF04JinJu) and the new LCDV isolate from sea bass, SB98Yosu, were employed in the present study. The first two letters of each of the isolate names, JF, RF, and SB, indicate the host fish, Japanese flounder, rockfish and sea bass, respectively. The numbers designate the year of isolation, and the letters following the numbers represent the areas in which the fishes were collected; Yosu, GunNe, ShinJi, Jeju, and JinJu denote names of cities in the southern part of Korea, whereas Yoshi and Kuma are abbreviations of Yoshioka and Kumaishi in Hokkaido, Japan. Lymphocystis cells from LCD fish were homogenized in 10 volumes of Hanks' balanced salt solution (HBSS), and centrifuged at 2,000 × g at 4°C. The supernatants were collected as an LCDV suspension, and stored at −80°C until use.

In order to clarify the genetic position of the SB98Yosu isolate in the genus *Lymphocystivirus*, the MCP gene in the isolate was sequenced using an LC1-F&R primer set, as described by Kitamura *et al.*, (2006). The determined nucleotide sequence was assembled using the Genetyx-Win program (Ver. 5.1), and multiple alignments were constructed with the Clustal X program (Thompson *et al.*, 1997) to find an optimal phylogenetic tree, using neighbor-joining criteria. The final phylogenetic tree was drawn using the NJplot program (Perrière and Gouy, 1996). For comparative purposes, the MCP gene nucleotide sequences of twelve lymphocystiviruses, LCDV-1 (genotype I), Japanese flounder isolates (genotype II) and three rockfish isolates (genotype III), were downloaded from the DNA data bank of Japan (DDBJ).

Nucleotide sequence analyses showed that the PCR product of the SB98Yosu isolate was 1,356 bp long, and coded for 451 amino acid residues (data not shown). The sequence determined in this study was registered in the DDBJ under the accession number AB247938. Comparison of sequence identities between SB98Yosu and eight Japanese flounder isolates,

Table 1. Sequence identities in nucleotide and amino acid levels of major capsid protein

Isolates	lymphocystiviruses							
	LCDV-1		LCDV-C		SB98Yosu		RF04Yosu	
	nt (%)	a.a. (%)	nt (%)	a.a. (%)	nt (%)	a.a. (%)	nt (%)	a.a. (%)
LCDV-1	100	100	78.8	87.3	79.7	88.7	81.1	87
LCDV-C	78.8	87.3	100	100	90.3	96.5	85.2	92.2
LCDV-JF00Yosu	78.8	87.3	100	100	90.3	96.5	85.2	92.2
LCDV-JF03GunNeA	78.8	87.3	100	100	90.3	96.5	85.2	92.2
LCDV-JF03ShinJi	78.6	87.3	99.9	100	90.1	96.5	85	92.2
LCDV-JF03GunNeB	78.8	87.3	100	100	90.3	96.5	85.2	92.2
LCDV-JF04Jeju	78.6	87.1	100	100	90.3	96.5	85.2	92.2
LCDV-JF03Yoshi	78.6	87.1	99.7	99.6	90.1	96.5	85.2	92.2
LCDV-JF00Kuma	78.5	86.8	99.7	99.3	90.1	96.5	85	92
LCDV-SB98Yosu	79.7	88.7	90.3	96.5	100	100	85.8	93.1
LCDV-RF03Yosu	81.1	87	85.2	92.2	85.8	93.1	100	100
LCDV-RF04Yosu	81.1	87	85.2	92.2	85.8	93.1	100	100
LCDV-RF04JinJu	81.1	87	85.2	92.2	85.8	93.1	100	100

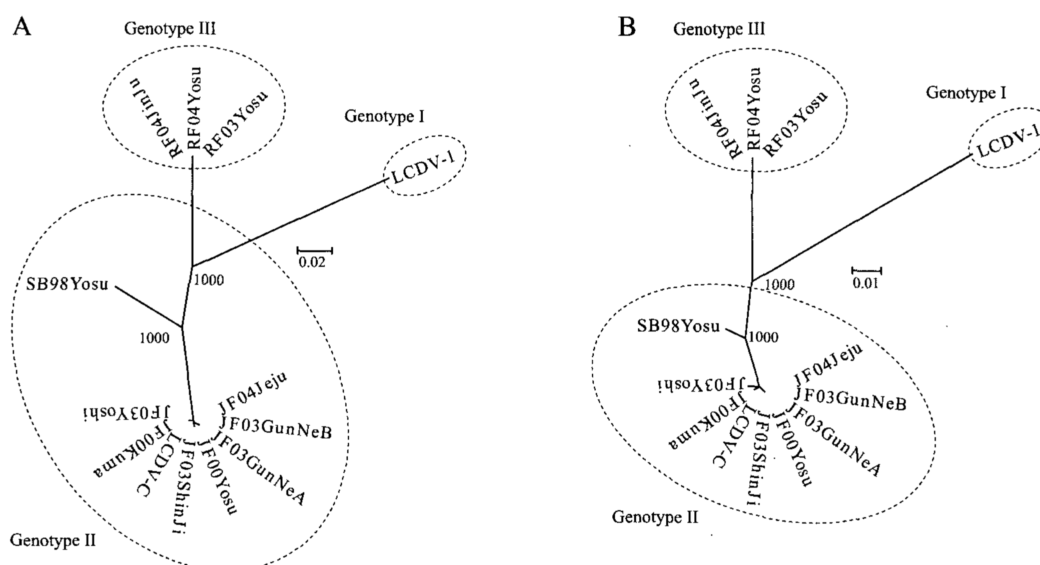

Fig. 1. Molecular phylogenetic tree representing the genetic relationship between 13 lymphocystivirus isolates on the basis of major capsid protein (MCP) gene nucleotide (A) and amino acid sequences (B). Bootstrap values at 1,000 times construction are shown at major nodes on the tree. The scale bar is for a genetic distance marker (number of replacement nucleotides per site). The Genbank accession numbers for the nucleotide sequences are as follows: SB98Yosu (AB247938), LCDV-1 (L63545), LCDV-C (AY380826), JF00Yosu (AB212999), JF03GunNeA (AB213001), JF03GunNeB (AB213002), JF03ShinJi (AB213000), JF04Jeju (AB213003), JF00Kuma (AB212997), JF03Yoshi (AB212998), RF03Yosu (AB213004), RF04Yosu (AB213005) and RF04JinJu (AB213006).

revealed identity of more than 90.1% at nucleotide level and 96.5% at deduced amino acid level, respectively, however, SB98Yosu and rockfish isolates or LCDV-1 showed 85.8% sequence identity or less at nucleotide level (Table 1). A phylogenetic tree based on the MCP gene nucleotide sequences and amino acid sequences revealed the following three major clusters described previously by Kitamura *et al.*, (2006): the first cluster (genotype I) included a single strain, LCDV-1; the second cluster (genotype II) comprised eight isolates from Japanese flounder; and the third (genotype III) consisted of three isolates from rockfish (Fig. 1A & B). The SB98Yosu isolate, newly sequenced in this study, appeared in genotype II along with the Japanese flounder isolates. Wolf (1988) previously reported that LCDV is best replicated by cell cultures derived from either the host or from a closely-related fish species. It was, therefore, interesting to find that the sea bass isolate (SB98Yosu) was closely related to the Japanese flounder isolates (JF-series), although these two host fish species are classified biologically into different orders, i.e., Japanese flounder belongs to the pleuronectiformes and sea bass belongs to the perciformes. Thus, further investigations into the relationship between the genetic diversity of LCDV and host fish species were needed.

For the multiplex PCR performed in this study, the primer sets were designed on the basis of the MCP gene sequence information, and their positions on the MCP gene are shown in Fig. 2. Three hyper-variable regions were selected for the design of the forward primers (GI, GII and GIII), and one reverse primer (LCDVs-R) was designed on a region that was conserved among these viruses. Also, one universal forward primer (LCDVs-F), which was designed to detect all of these viral genotypes, was based on the region conserved among the lymphocystiviruses. The primer sequences were as follows: 5'-YTGGTTCAGT AAATTACCRG-3' - LCDVs-F; 5'-GTAATCCATACTT GHACRTC-3' - LCDVs-R; 5'-TTAGATTATTGGGCA GCGTT-3' - GI; 5'-TYGATTCCAAYGGTCAATTA-3' - GII; 5'-AGGAAATAACAACCGTATGAATGCA-3' - GIII. The expected sizes of the PCR products were 341 bp for genotype I, 250 bp for genotype II, 468 bp for genotype III, and 609 bp for all genotypes.

The LCDV genomic DNA was extracted from the stored virus suspensions, as was previously described by Kitamura *et al.*, (2006). In brief, a 20 μl aliquot of proteinase K (1 mg/ml; TaKaRa, Japan) was added to 200 μl of the LCDV suspension. This mixture was then incubated for 2 hours at 55°C. The DNA was isolated with phenol and chloroform. The nucleic acids were precipitated using isopropanol, resuspended with distilled water, and stored at –20°C until use. Initially, the specificity of each of the primer sets was tested. PCR amplification was conducted using 20 μM of primers and an AccuPower^{TM} PCR premix kit

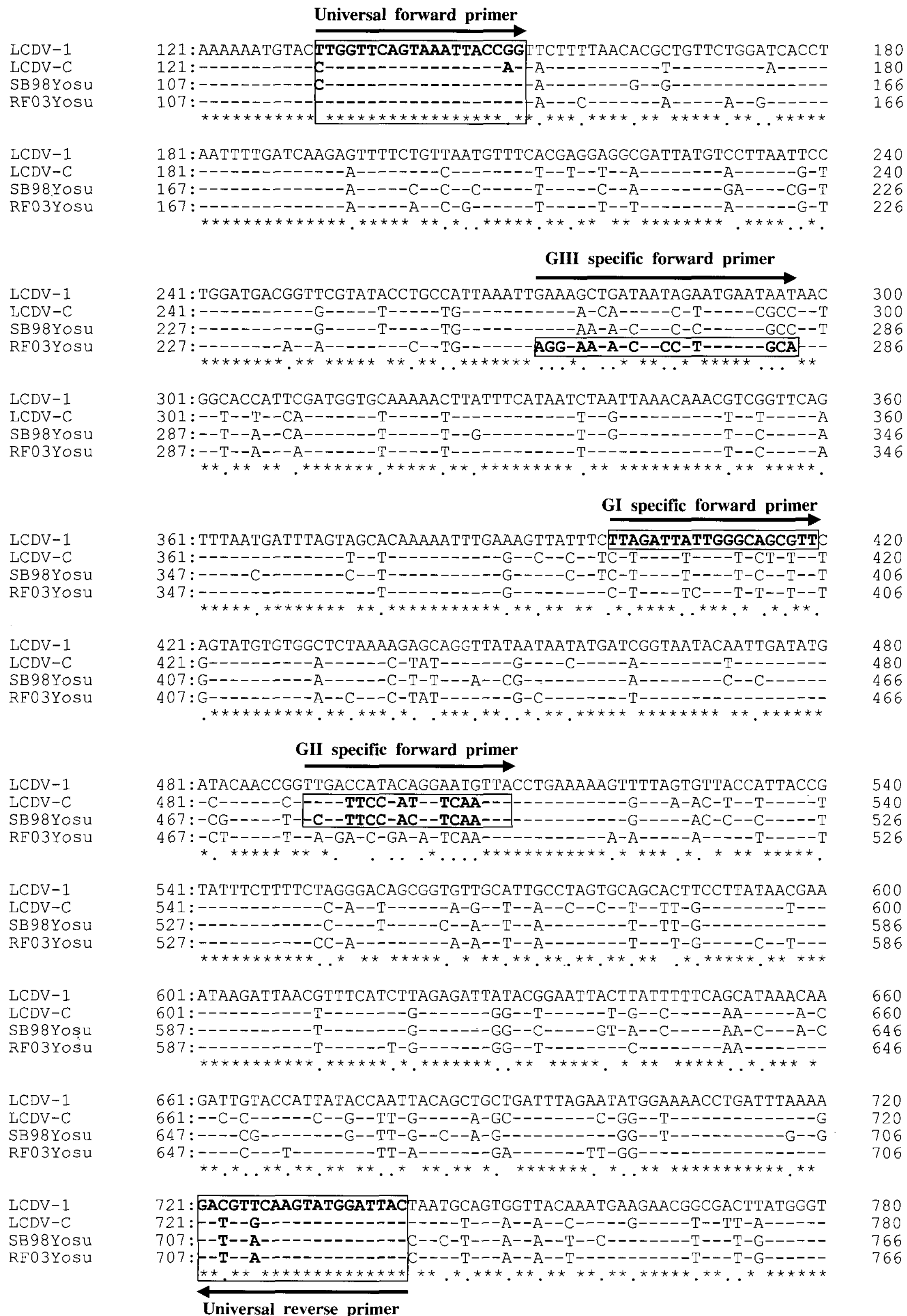

Fig. 2. The nucleotide sequence alignment of major capsid protein genes from four lymphocystiviruses. The Genbank accession numbers for the nucleotide sequences are as follows: LCDV-1 (Tidona and Darai, 1997; L63545), LCDV-C (Zhang *et al.*, 2004; AY380826), SB98Yosu (in this study; AB247938) and RF03Yosu (Kitamura *et al.*, 2006; AB213004). Hyphens indicate the same nucleotides as LCDV-1. Four forward primers (LCDVs-F, GI, GII and GIII) and one reverse primer (LCDVs-R) used for PCR are shown in boxes.

(Bioneer, Korea), in accordance with the manufacturer's instructions. Secondly, for the multiplex PCR, 20 μM of each of the primers (GI, GII and GIII forward primers and LCDVs-R reverse primer) was mixed and subsequently employed in the reactions. All PCR amplifications in this study were conducted with a GeneAmp 2400 thermal cycler (Perkin Elmer, USA) for 30 cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min). The PCR products were analyzed on 2% agarose gels containing ethidium bromide, and visualized under UV light.

As is shown in Fig. 3, a PCR product of 609 bp was obtained from three isolates, including JF03GunNeA (genotype II), SB98Yosu (genotype II) and RF03Yosu (genotype III) using the LCDVs-F and LCDVs-R universal primer set. Unfortunately, although no LCDV -1 from genotype I was available for analysis in this study, our results indicated that the universal primer set could be of use in the detection of at least two LCDV genotypes: genotype II (Japanese flounder and sea bass isolates) and III (rockfish isolates). Also, the PCR reaction gave rise to highly specific PCR products for the individual isolates, including JF03GunNeA (250 bp), SB98Yosu (250 bp), and RF03Yosu (468 bp), using GII or GIII forward primers and reverse LCDVs-R primer (Fig. 3). Specific PCR products were also obtained from other LCDV isolates, including JF00Yosu, JF03GunNeB, JF03ShinJi, JF04Jeju, JF00Kuma, and JF03Yoshi isolated from Japanese flounder, and RF04Yosu and RF04JinJu isolated from rockfish (data not shown). In addition, no PCR amplification was observed in any of the isolates belonging to genotypes II or III when the GI forward primer and the LCDVs-R reverse primer were used (Fig. 3), although it remains unclear as to whether this primer set is capable of amplifying specific PCR products from the LCDV-1 genome. Thus, our results strongly suggest that the primer sets utilized in this study can, at least, distinguish clearly between LCDV isolates from genotypes II and III. In the multiplex PCR, specific single bands were amplified when the individual genome of each isolate was employed as the DNA template (Fig. 4), indicating that the mixing of three forward primers has no effect on the specificity of the PCR. In order to evaluate the practical applications of this method in the detection of LCDV, genomic DNA prepared from two or three LCDV isolates (JF03GunNeA, SB98Yosu and RF03Yosu) were mixed and subjected to multiplex PCR. Amplicons corresponding to each of the isolates were then generated from the mixed DNA specimens (Fig. 4). Thus, the multiplex PCR based on the MCP gene had a capacity to distinguish at least two viral genotypes, even in a sample including more than 2 viral genotypes.

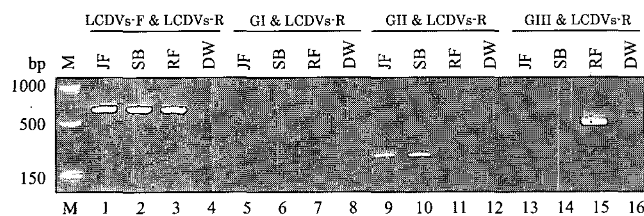

Fig. 3. Specific PCR analysis of three LCDV isolates. Lane M: molecular weight marker. Lanes 1-4: reaction performed with the LCDVs-F and LCDVs-R primer set. Lanes 5-8: reaction performed with the GI and LCDVs-R primer set. Lanes 9-12: reaction conducted with the GII and LCDVs-R primer set. Lanes 13-16: reaction performed with the GIII and LCDVs-R primer set. JF, SB, RF and DW indicate JF03GunNeA, SB98Yosu, RF03Yosu and distilled water, respectively.

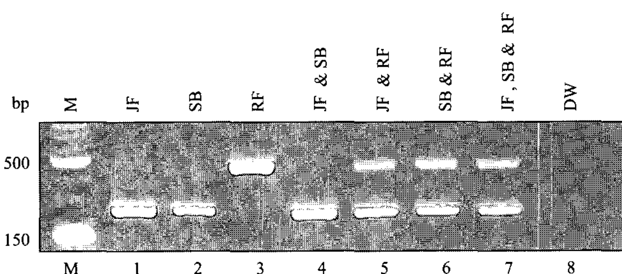

Fig. 4. Multiplex PCR using genomic DNA from three LCDV isolates. Lane (M) Molecular weight marker. (1) JF03GunNeA, (2) SB98Yosu, (3) RF03Yosu, (4) JF03GunNeA and SB98Yosu, (5) JF03GunNeA and RF03Yosu, (6) SB98Yosu and RF03Yosu, (7) JF03GunNeA, SB98Yosu and RF03Yosu, (8) Distilled water used as a negative control.

With regard to LCDV detection, serological techniques including indirect immunofluorescence and flow cytometry, using an anti-LCDV polyclonal antibody, have been reported to be effective (García- Rosado *et al.*, 2002). DNA sequencing undoubtedly provides the most accurate information for LCDV genotyping (Kitamura *et al.*, 2006), but is not currently available as a routine diagnostic procedure. This method is also both expensive and time-consuming, as compared to non-sequencing methods. Compared to these methods, multiplex PCR has the advantage of being simple, fast, and requiring no specialized laboratory equipment other than the standard PCR instruments. One of the disadvantages associated with this technique is the necessity for manual handling prior to the visualization of PCR products. A minimum of handling or, at best, no handling of the PCR products would be preferable, as the primary concern regarding diagnostic PCR is the risk of contamination of new samples with the PCR products from previous reactions (carry-over contamination).

Thus, in our multiplex PCR assay, we have developed a method for the detection and differentiation of LCDV isolates, which can be applied in any laboratory containing standard PCR equipment. Although

the transmission modes of LCDV have yet to be definitively elucidated, effective disease control will rely on our understanding of the dynamics of the virus in host fish species and environmental waters. Based on the results presented here, we believe that the primer sets designed in this study will constitute the most valuable of the available tools for the evaluation of LCDV distribution.

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