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Cloning and Sequence Analysis of Capsid Protein Gene of Iridovirus Indonesian Isolates

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Abstract

Iridovirus was known as agents that caused serious systemic disease in freshwater and marine fishes. The mortality up to 100% of orange-spotted grouper (*Epinephelus coioides*) due to iridovirus infection has been reported in Indonesia. The gene encoding capsid protein of iridovirus is supposed to be conserved and has the potency for the development of control methods. The objectives of this study are to clone the gene encoding capsid protein iridovirus and to analyze their sequences. The spleen tissues of orange-spotted grouper were collected and extracted their DNA. The DNA fragment of capsid protein of iridovirus genes were amplified by PCR using designed primers with the extraction DNA as templates. The amplified DNA fragments were cloned in pBSKSII and sequenced. The genes encoding capsid protein of iridovirus from Jepara and Bali were successfully amplified and cloned. The Jepara clone (IJP03) contained complete open reading frame (ORF) of the gene composed by 1362 bp nucleotides which encoded 453 amino acids. Those Jepara and Bali (IGD01) clones shared 99.8% similarity in nucleotide level and 99.4% at amino acid level. Based on those sequences, Indonesian iridovirus was belonged to genus Megalocystivirus and shared 99,6-99,9% similarity on nucleotide level with DGIV, ISKNV, MCIV, and ALIV.

Keywords : cloning, sequence analysis, capsid protein gene, iridovirus

Introduction

Grouper is an important fish species due to the high price both in domestic and international markets, especially in alive condition (Murdjani, 1999). However disease is a major cause of loss and low survival rate of larvae. Nodavirus and iridovirus infections were often found with cases of mass mortality of grouper during larvae rearing (Aslianti and Priyono, 2006). Iridovirus has been found as diseases on orange-spotted grouper (*Epinephelus coioides*) is North Sumatra wich caused mortality more than 80%. This virus was also detected in Bali on orange-spotted grouper (*E. coioides*) and duskytail grouper (*E. bleekery*) during acclimatization after being caught from the sea, with mortality up to100% (Roza *et al*., 2005).

The clinical signs of iridovirus-infected fishes were severe anemia, red spots (ptechiae) in the gills, swelling of the spleen (Nakajima and Maeno, 1998) and kidney (Sudthongkong *et al*., 2002). Under histopathology examination, inclusion body forming bearing cells (IBC) were found in spleen, kidney, hematopoietic tissue and the digestive tract, and necrosis was occurred in kidney tissue (Sudthongkong *et al*., 2002).

Iridovirus a double-stranded DNA virus, icosahedral symmetry shaped with 120-300 nm in diameter (Murphy *et al*., 1995).

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This virus has a vary genome between 105-212 kbp (Lu *et al*., 2002), 25-75 structural proteins with molecular weight 12 -150 kDa (Kalmakoff, 1998). Generally the protein capsid size is about 50 kDa and posed major structural component about 45% of the total virion proteins (Black *et al*., 1981).

The genetic diversity of iridovirus has been analyzed based on capsid protein such as by Webby and Kalmakoff (1998) among 18 isolates iridovirus; by Sudthongkong *et al.* (2002) among 5 isolates of tropical iridovirus, and by Do *et al.* (2005) among 10 isolates in Korea. This molecular analysis is important for the development of recombinant vaccines (Caipang *et al*., 2006) and detection methods (Chao *et al*., 2002). However, the molecular information of this virus which infected grouper in Indonesia has not been reported. This study was conducted to clone and analyze the gene encoding capsid protein of iridovirus Indonesian isolates.

Materials and Methods

DNA Extraction

Spleen tissues were collected from the orange-spotted grouper showing symptoms infection of iridovirus from Bali (IGD) and Jepara (IJP). The extraction of DNA from spleen tissues referred to the method developed by Wasko *et al.* (2003). The 10 - 30 mg of spleen tissue was homogenized in 400 ml TNES buffer (10 mM Tris-HCl pH 8; 125 mM NaCl; 10 mM EDTA pH 8; 0,5% SDS; 4M urea). A 3 ml of RNase (10 mg/ml) was added to the mixture and followed by incubation at 42°C for 1 h. After this incubation 3 ml of proteinase K (10 mg / ml) was added into the mixture and incubated again at 42°C for 2-6 h. The suspension was extracted using same volume of phenol: chloroform: isoamyl alcohol (PCIAA) and DNA was precipitated using 1 M NaCl and two times the volume of cold absolute ethanol. The DNA was washed with 70% ethanol and dissolved in TE buffer.

Amplification

DNA fragment of capsid protein of iridovirus gene was amplified using polymerase chain reaction (PCR) method (Sambrook and Russell, 2001) with a thermocycler GeneAmp PCR System 2400 (PE Applied Biosystems). Primers were designed to amplify the ORF capsid protein fully or partially, those are Irido-MCP-F-Bam: 5'-ATCAGGATCCATGTCTGCAATCTCAGG TG-3', Irido-MCP-R-Eco: 5'-CGTCGAATTCGTCGACAGATGTGAAG TAG-3', and Irido-MCP-2R-Eco: 5'-CGTCGAATTCTTACAGGATAGGGAAG-3'. Amplification was carried out using Taq DNA polymerase and other reagents (Promega) in following conditions: pre-denaturation at 94°C for 2 min, followed by 30 amplification cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 75 sec (IGD01 sample) or 90 sec (IJP03 sample) and with extra extension at 72°C for 5 min. A small portion (5 ul) of PCR products were applied on agarose electrophoresis to ensure the amplification process.

Cloning

The PCR products were purified using phenol-chloroform method (Sambrook & Russell, 2001) and continued with digestion using *Eco*RI and *BamH*I. The pBSKSII vector was also digested with the same enzymes. After digestion, DNAs were electrophoreted, then DNA vector and PCR products were purified from agarose with a glass powder method (modification of Sambrook and Russell, 2001). The digested PCR products and vector were quantified by spot test, comparing the intensity of sample with DNA standards in the presence of ethidium bromide on UV transiluminator.

Ligation of PCR product on vector

cloning was done in proportion vector and insert as 1:3 using T4 DNA ligase (Toyobo) at 16°C for 24 h. Transformation of ligation mixture was performed on *E. coli* DH5a using heat shock method (Sambrook and Russell, 2001). Ligation solution was mixed with competent cell bacteria and put in ice for 10 min, incubated on waterbath at 42°C for 90 sec and immediately put into ice. Bacteria were plated on LB Agar 50 mg/ml amphicilin and incubated at 37°C for over night. The bacteria colonies which appeared were cultured on LB broth containing 50 mg/ml amphicilin for over night. The plasmids were isolated from bacteria using alkali lysis method (Sambrook and Russell, 2001). The plasmids were electrophoreted to seek the possibility plasmid carrying the insert (recombinant) which showing larger size than the control plasmid. To make sure the presence of desired insert, the plasmids were digested using one or two restriction enzymes (EcoRI and / or BamHI).

Sequencing and data analysis

Plasmids carrying desired insert were purified and sequenced using T3 and T7 primers using ABI Prism 3100-Avant Genetic Analyzer provided by a company. BLAST analysis was performed to determine the sequence similarity with the data in Genbank. Alignment analysis between the sequences data was performed with ClustalW program. Multiple alignments for phylogenic analysis of the collected data from Genbank was done using the CLC Free Workbench program 3. Construction of phylogenetic trees was based on unweighted pair group method using arithmetic average (UPGMA) and bootstrap analysis was performed with resampling 1000 times.

Results and Discussion

The result of this study proved that the orange-spotted groupers which used in this study were infected by Iridovirus as indicated by presence a band of DNA as product of amplification using specific primers for iridovirus capsid protein. The size of amplification product of the gene was 1000 bp from IGD01 samples (isolate Bali, Figure 1, lane 1) using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco. The larger size of DNA band was found from amplification product of samples IJP03 (isolate Jepara) using primers MCP-Irido-F-Bam and MCP-Irido-2R-Eco as 1350 bp (Figure 1, lane 2).

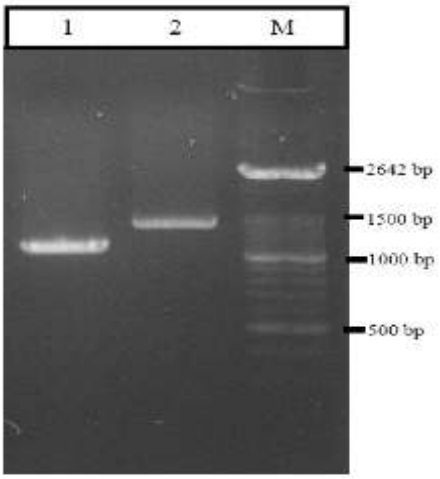

Figure 1. DNA fragments as products of amplification of iridovirus protein capsid gene. 1. Bali sample was amplified using MCP-Irido-F-Bam and MCP-Irido-R-Eco primers. 2. Jepara sample was amplified using MCP-Irido-F-Bam and MCP-Irido-2R-Eco. M. the 100 bp DNA Ladder with indicated size.

Plasmids were isolated from colonies of *E. coli* which grown in LB medium containing amphicilin and electrophoreted in agarose. The presence of circular plasmid larger (Figure 2, lane 2) from the plasmid control (non-recombinant) (Figure 2, lane 1) as shown in Figure 2, showed the posibility the plasmid as a recombinant plasmid. To verify as a recombinant, plasmid were digested *Eco*RI, and *BamH*I.

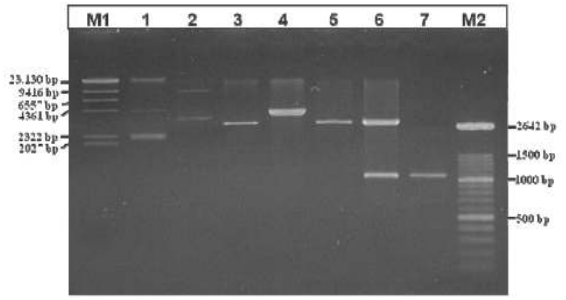

Figure 2. Verifying the recombinant plasmid. 1. Undigested pBSKSII. 2. Undigested recombinant plasmid IGD01. 3. *Eco*RI digested of pBSKSII. 4. *Eco*RI digested of recombinant plasmid IGD01. 5. *Eco*RI and *Bam*HI digested of pBSKSII. 6. *Eco*RI and *Bam*HI digested of recombinant plasmid IGD01. 7. PCR product Bali sample using Irido-MCP-F-Bam and Irido-MCP-R-Eco primers. M1. ëDNA / *Hind*III marker and M2. 100 bp DNA Ladder marker with indicated sizes.

The digestion result showed the plasmid carrying capsid protein of iridovirus (IGD01 sample) was clearly different compared to control plasmid. After digestion with *Eco*RI, the position of recombinant plasmid band was approximately 4000 bp (Figure 2, lane 4). This position was higher than the position of control plasmid band as approximately 2900 bp (Figure 2 lane 3). After the recombinant plasmid was digested with *Eco*RI and *Bam*HI, two bands were appeared as approximately 2900 bp indicating a cloning vector and 1083 bp indicating a DNA insert (Figure 2, lane 6), a same size with the PCR product using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco (Figure 2, lane 7). The similar pattern was found using isolate Jepara (IJP03) using MCP-Irido-F-Bam and MCP-Irido-R2-Eco except the size of DNA insert was higher as approximately 1350 bp. Those results indicated that iridovirus capsid protein genes were successfully cloned form Bali and Jepara isolates. The few researches have been done on iridovirus in Indonesia. Most of them were analysis on pathogenesis, pathology and detection (Roza *et al.*, 2005; Aslianti & Priyono, 2006). So this study is the first time research on molecular biology of iridovirus in Indonesia by cloning and sequence analysis of iridovirus gene.

The complete ORF of the gene was successfully cloned from Jepara sample (IJP03 clone) composed by 1362 nucleotides and encoded 453 amino acids. The partial ORF of the gene was cloned from Bali sample (IGD01 clone) only contained 1083 nucleotides. Alignment analysis showed that those two clones showed very high similarity as 99.8%. Nucleotide substitutions were only occurred only in 4 positions 43, 178, 548 and 877 (Figure 3).

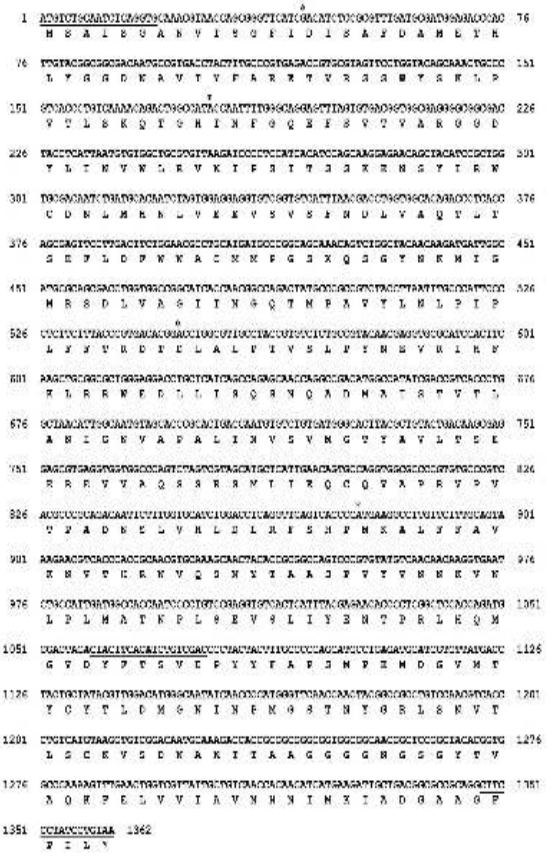

Figure 3. The open reading frame (ORF) of iridovirus capsid protein. The IJP03 clone contains complete ORF contains 1362 nucleotides from start codon ATG to the stop codon TAA. The IGD01 clone only contains nucleotide 1 - 1083. The four nucleotide substitutions IJP03 to IGD01 are indicate by asterisk (*) with GàA , AàG , AàG , AàG. The underline nucleotides indicate the primers position used in this study.

Those nucleotide substitutions produced 4 amino acids substitutions. Nucleotide BLAST analysis showed that capsid protein Jepara and Bali clones have similarity at nucleotide level as 99,6-99,9% for *dwarf gouramy iridovirus* (DGIV) (access no AY989901), *infectious spleen and kidney necrosis virus* (ISKNV) (access no AF371960), *Murray cod iridovirus* (MCIV) (access no AY936203), and *african lampeye iridovirus* (ALIV) (access no AY285745). Those high similarity results indicated that gene coding for the capsid protein genes were conserved.

In 2003, a new family Iridoviridae classification was proposed to ICTV and dividing this family into 5 genera, including: Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, and Megalocystivirus. Phylogenetic analysis on partial capsid protein ORF (1077 nt) from 20 isolates accessed from GenBank which representing those five genera showed that IGD01 and IJP03 clones were belonged to genus Megalocystivirus (Figure 4). This result showed that orange-spotted grouper (*Epinephelus coioides*) which used in this study were infected by Megalocystivirus. This *Megalocystivirus* was known as agents that caused serious systemic disease in freshwater and marine fishes (Lu *et al*., 2005). This virus have been reported cause disease on *Epinephelus sp.* (Chou *et al*., 1998), *E. tauvina* (Chua *et al*., 1994), *E. malabaricus* (Danayadol *et al*., 1996), *C. lalia* (Sudthongkong *et al*., 2002), dan *S. maximus* (Shi *et al*., 2004).

Genus Megalocystivirus have been reported infect fishes form Korea, China, Jepang, Thailand, Singapura, and Australia. Go *et al*. (2006) stated that this iridovirus is predicted originally from tropic Asia and spreaded to other country though trading of food and ornamental fishes.

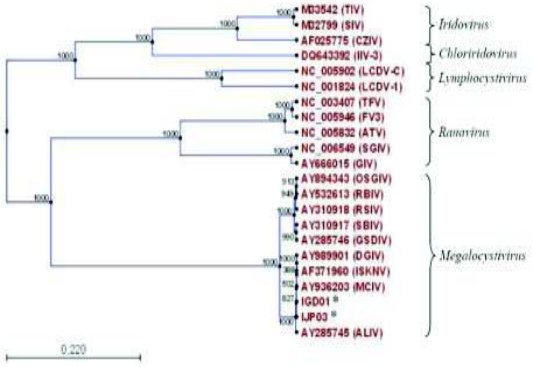

Figure 4. Molecular phylogenetic tree of family Iridoviridae Nucleotide sequence analysis of genes coding for partial capsid protein (nt 1077), including 20 isolates that are accessed from GenBank and 2 isolates from this study (*). Scale length of the horizontal line shows the proportional to the number of nucleotide substitution. Numbers at branching points indicate bootstrap values from sampling 1000 times.

Phylogenetic analysis on both Indonesian iridovirus clones is located on the same branch with the iridovirus isolates which infect freshwater ornamental fishes, *Colisa lalia* (AY989901) *Maccullochella peelii peelii* (AY936203) and *Aplocheilichthys normani* (AY285745). This result supported the possibility of iridovirus spreading was through ornamental fish trading.

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