MHC-II gene and its association with disease resistance to koi herpes virus in five strains of common carp

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Abstract. Koi herpes virus (KHV) disease is one of the main problems in common carp (*Cyprinus carpio*) culture in Indonesia. In teleost fish, Major Histocompatibility Complex class II (MHC-II) gene was suggested to be associated with resistance to this disease. Identification of KHV resistant common carp can be done through marker assisted selection (MAS) targeting MHC-II gene. This study aimed to analyze the genetic of five common carp strains using MHC-II molecular markers and to evaluate their resistance to KHV. The five stocks of common carp which were dominantly cultured by farmers, namely strain of Rajadanu, Sutisna, Majalaya, Wildan and Sinyonya were used in this study. The presence of MHC-II genes especially the allele of Cyca-DABI*05 in these strains were detected using PCR method. Evaluation of resistance to KHV disease was performed through cohabitation challenge test. To evaluate the phenotypic performance especially in growth, all strains were reared in ponds for 90 days. The results showed that the Rajadanu and Wildan strains had 90% of individual carriers of MHC-II gene, and were higher than other strains. Results of the challenge test with KHV showed that Rajadanu strain had the highest survival rate (36.7%), followed by Majalaya, Sinyonya, Sutisna and Wildan strains, which reached 26.7%; 23.3%; 18.9% and 13.3%, respectively. The lowest survival rate of Wildan strain was suggested to be related to the growth characteristic of the fish that is relatively faster than other strains.
Key Words: common carp, resistance to KHV, Marker Assisted Selection, MHC-II.

Introduction. Common carp (*Cyprinus carpio* Linn., 1758) culture in Indonesia has a very serious problem due to Koi Herpes Virus (KHV) disease outbreak since 2002 (Sunarto 2005). The outbreak caused a significant decrease in production of common carp farming (Rukmono 2005). Efforts have been made to overcome the disease such as environmental improvement and externally resistance improvement of the common carp. The external resistance improvements were done by provision of vaccines (Taukhid et al 2005) and imuno stimulants from plants (Mudjiutami et al 2007) and other materials such as the chromium yeast (Zainun 2007). However, efforts to improve the resistance through genetic improvements of common carp, such as by selection program, have not been done yet.

One of the widely used selection methods in the last decade was the selection based on molecular markers called Marker Assisted Selection (MAS). MAS become an effective selection method as this method can be done without killing the biological product. The method has been used widely to select different traits such as disease resistance, edible portion and fillet quality, and feed efficiency (Sonesson 2007; Wakchaure et al 2015). In disease resistance improvement, Major Histocompatibility Complex (MHC) markers have been widely applied. Previous studies reported that MHC genes are generally associated with resistance to disease in vertebrates including fish (Hill et al 1991; Kaufman & Wallny 1996; Briles et al 1977; Rakus et al 2003; Liu et al 2014; Cao et al 2018). In teleost fish, the MHC gene was located in chromosome and associated with disease resistance properties (Grimholt et al 2003; Kjøglum et al 2006; Xu et al 2008). According to Rakus et al (2003), in common carp there were two classes of MHC-II gene genes, Cyca-DAB1-like genes, and Cyca-DAB3-like genes. This study indicated a high polymorphism of Cyca-DAB1-like, but not Cyca-DAB3. Furthermore Rakus et al (2009) showed an association between carp genotypes which had MHC-II gene with their resistance to CyHV (Cyprinid Herpesvirus) disease. The association between the MHC-II (Cyca-DAB1-like) genotype with high resistance to CyHV-3 indicated that selection based on marker associated to MHC-II gene could be used to improve the disease resistance in carp fish.

There are several strains of common carp which are dominantly cultured by farmers in Indonesia which showed different phenotypic performance, in term of growth and resistance to disease (Ariyanto et al 2014; Hayuningtyas et al 2015). These various strains with phenotypic variation are valuable materials for breeding programs. This study aimed to evaluate the resistance to KHV of five strains of common carp related to the presence of MHC-II gene, especially the Cyca-DAB1*05 allele. The result of this study will be useful to increase the success of selection for disease resistance improvement as a part of breeding programs of common carp in Indonesia.

Material and Method

Preparation of test fish. Five strains of common carp from the collection of Research Institute for Fish Breeding (RIFB), Sukamandi, West Java were used in this study. These strains were collected from the origin area during 2010-2012. They were Rajadanu and Sutisna strains from Kuningan regency (West Java), Majalaya strain from Bandung regency (West Java), Wildan strain from Cianjur regency (West Java) and Sinyonya strain from Pandeglang regency (Banten) (Figure 1). The collected fishes were ranging from 15 to 40 fishes of each strain with 350-5,500 g of body weight (Ariyanto et al 2014). Caudal fin was clipped from 7-10 fishes of each strain and were used as a random sample for MHC-II gene identification at laboratory.

Figure 1. Geographic area of the origin of five strains of common carp in Indonesia. RIFB: Research Institute for Fish Breeding. 1-2: Kuningan regency; 3: Bandung regency; 4: Cianjur regency; 5: Pandeglang regency.

Fishes from each sex were tagged with individual microchip tag and reared communally in a 400 m^2 of pond. The fishes were fed with commercial feed containing 38-40% of crude protein, at 2% day^{-1} based on body weight to optimize the maturity of the fish. Three pairs mature broodstock of each strain were spawned with artificial spawning method. A mixture of GnRH/dopamine antagonist was injected intramuscularly to induce and synchronize the ovulation and spermiation of the broodstocks. Gametes were collected by dry method to induce the fertilization uniformity. After gamete activation, eggs were spread out at “kakaban”, an artificial substrate which was made from palm tree fiber. Incubation was carried out in hatchery concrete tanks. Hatched fry were kept in large tanks until stocking at the stage of swimming-up larvae. The larvae were reared in outdoor concrete tanks for two months. About 100 fishes with 10-12 g of body weight from each strain were randomly taken from the tanks for challenge test at laboratory. Another 500 fishes from each strain also were randomly taken from the tanks for evaluation of the phenotypic performance of fish, especially for growth character.

Molecular analysis of MHC-II gene. Analysis of the MHC-II gene was carried out following the Protocol No. 01 of Common Carp Breeding Program in Indonesia (NBC 2012). In brief, genomic DNA from caudal fin of each individual sample was extracted using DNeasy Blood & Tissue Kits (Qiagen) following the protocol provided by the manufacturer. PCR was performed using Maxima Hot Start Green PCR master Mix (2X) kit (Fermentas, Thermo Scientific). MHC-II specific primers especially for Cyca-DAB1*05 allele were used for the amplification (Rakus et al 2008; Sucipto et al 2011). The β-actin of common carp gene with 300 bp fragment length was used as an internal control. Electrophoresis was performed on agarose gel with concentration of 1.5% in 1×TBE buffer. Electrophoresis was carried out using an electric current of 80 V for 35 minutes. After staining using etidium bromide, DNA was visualized using UV gel doc trans-illuminator.

Challenge test for KHV. The challenge test was conducted using cohabitation method following Protocol No. 03 of Common Carp Breeding Program in Indonesia (NBC 2012). In cohabitation method, infected fishes were required to transmit KHV to the test fishes. KHV infected fishes were produced by intramuscularly injection of homogenate filtrate of KHV. The homogenate filtrate was obtained from gill extraction of cultured carp which was positively infected by KHV. Injected fish showing inflammatory clinical symptoms of KHV and confirmed molecularly infected by KHV were used as KHV transmitter fish in challenge test. The presence of KHV infection in the KHV transmitter fish was analyzed in laboratory following Protocol No. 03 of Common Carp Breeding Program in Indonesia (NBC 2012). Genomic DNA from caudal fin of the fish was extracted using DNAzol (DNAzol® Reagan Invitrogen) following the protocol provided by the manufacturer. PCR was performed using RTG beads kit (GE Healthcare). KHV specific primer with 290 bp fragment length was used for the amplification (Yuasa et al 2005). Electrophoresis was performed on agarose gel with concentration of 1.5% in 1×TBE buffer. Electrophoresis was carried out using an electric current of 100 V for 40 minutes. After staining using etidium bromide, DNA was visualized using UV gel doc trans-illuminator.

Challenge test was conducted by rearing fish of each strain in 60 liters of aquarium with a density about 30 fishes per aquaria. Fish were acclimated in aquarium for three days before the KHV transmitter fishes were stocked. The water temperature of aquarium was adjusted to the optimum temperature for KHV developed well, ranging from 20 to 22°C. The challenge test for KHV was conducted for 21 days with three replicates. The presence of KHV infection in the test fish was proven by taking samples of the fish showing clinical symptoms of KHV and molecularly analyzed in the laboratory following Protocol No. 03 of Common Carp Breeding Program in Indonesia (NBC 2012).

Evaluation of phenotypic performance. Phenotypic performance, especially in growth character was evaluated through rearing 150 fishes with 10-12 g of body weight from each strain in 3×5 m^2 of net cage for 90 days. There were three replicates. The fish were fed with commercial pellet feed containing 28-31% of crude protein. At the end of rearing period, all fishes were harvested and sampled for body weight and length. All live fishes were calculated for survival rate analysis and to estimate the biomass of the fish.

Statistical analysis. The percentage of individual fish carrying MHC-II markers on each strain was analyzed descriptively. Both of fish mortality in challenge test and the phenotypic performance of fish in net cages data were tested for assumptions of normality and homoscedasticity by the Bartlett's test and were log-transformed when required. Variations between strains were compared using one-way ANOVA, followed by Duncan's multiple-range test. Significance of differences was defined at $p < 0.05$. Statistical analyses were performed using SPSS® software.

Results

Molecular analysis of MHC-II gene. The presence of MHC-II genes in five strains of common carp were presented in Figure 2, whereas the percentage of individual fish carriers the MHC-II genes was presented in Table 1.

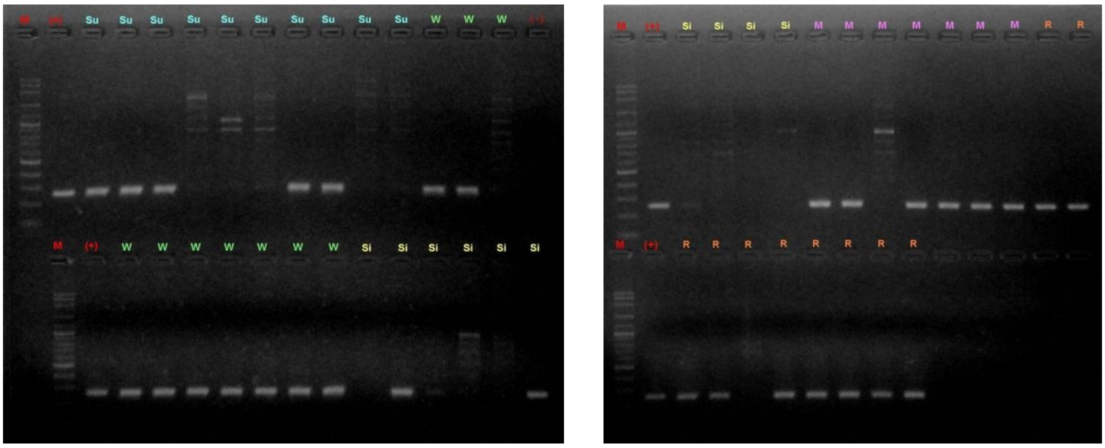

Figure 2. Molecular analysis of MHC-II gene in each strains of common carp (Su: Sutisna; W: Wildan; Si: Sinyonya; M: Majalaya and R: Rajadanu).

The results showed that Cyca-DAB1*05 allele in five strains of common carp has appeared in 300 bp region (Figure 2). Several other studies have shown different lengths of the Cyca-DAB1*05 allele, such as 260 bp (Alimuddin et al 2011), 500 bp and 1000 bp (DCA 2010; Rina 2014). The previous studies reported that Cyca-DAB1*05 allele in common carp was generally polymorphic, but we found that all alleles of common carp in this research were monomorphic.

Table 1

Percentage of individual fish carriers the MHC-II gene in five strains of common carp

No.	*Strain*	*Fish number*	*Fish with MHC-II gene number*	*Percentage (%)*
1.	Rajadanu	10	9	90
2.	Sutisna	10	5	50
3.	Majalaya	7	6	86
4.	Wildan	10	9	90
5.	Sinyonya	10	4	40

The results showed that Rajadanu and Wildan strains have more MHC-II genes compared to others. About 90% of both populations were carrying MHC-II genes (Table 1). It were indicated the high levels of resistance to KHV in both strains. The population with less number of individuals carrying MHC-II markers was Sinyonya strain that were only 40% of population. The low percentage of individual carrying the gene encoding resistance to KHV indicated a low level of resistance of Sinyonya strain to KHV disease.

Challenge test. Molecular confirmation of KHV infection in the KHV transmitter fish is presented in Figure 3. The results of molecular analysis clearly showed that the transmitter fishes which injected intramuscularly by filtrate homogenate KHV were positively infected by KHV (290 bp). The positive KHV fish based on lab analysis were then cohabited with the test fish as KHV transmitter fish.

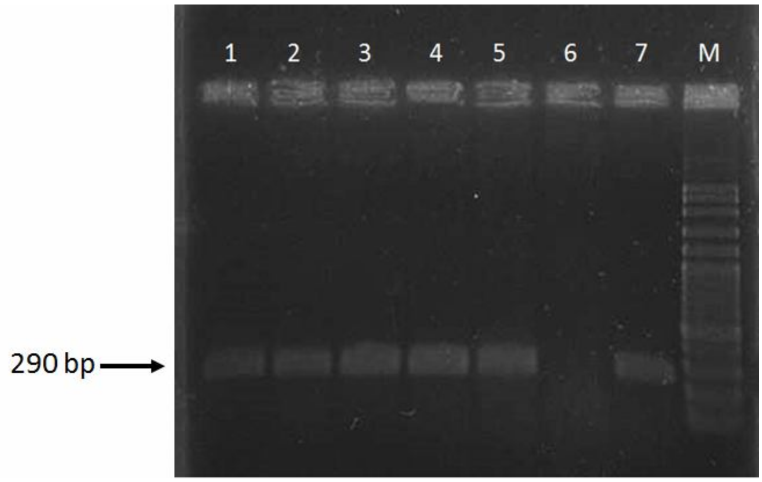

Figure 3. Molecular KHV confirmation result for the transmitter disease fish. 1-5: sample fish; 6: control (-) fish; 7: control (+) fish; M: marker.

The accumulated fish mortality in the end of challenge test is presented in Table 2. In general, the mortality rate of test fish that was cohabited with KHV transmitter fish was significantly different from negative control of fish, which is not infected with KHV. This result indicated that the death of fishes in the challenge test aquaria were caused by KHV which infected by the KHV transmitter fish. KHV disease attack since 6th day after the test fish were cohabited with KHV transmitter fish in aquaria. The disease was reached the peak at 7th – 10th day of cohabitation. The number of mortality fish was reduced after the 10th day. The results of this experiment are consistent with the results of the previous experiments (Shapira et al 2005; Novita & Koesharyani 2005; Ødegård et al 2010) which indicated that the peak of KHV attacks usually occurs on the day 5th to the 8th. Table 2 showed that accumulated dead fish number was significantly different between strains. The highest fish mortality number occurred in Wildan strain, which reached 26 of 30 fishes. It was significantly different from other strains. The lowest mortality fish number was occurred in Rajadanu strain, which is also significantly different from other strains.

Table 2

Fish mortality accumulation of five strains of common carp during challenge test with KHV

Strain	*Fish test number (individual)*	*Dead fish number (individual)*
Control population	30	0.0 ± 0.0^{a}
Rajadanu	30	19.0 ± 1.0^{b}
Sutisna	30	24.3 ± 1.5^{cd}
Majalaya	30	22.0 ± 1.0^{c}
Wildan	30	26.0 ± 1.0^{d}
Sinyonya	30	23.0 ± 1.0^{c}

The significance values (p > 0.05) were showed by different superscript letters.

The results of molecular confirmation of KHV infection in fish from challenge test is presented in Figure 4. The results of the confirmation have a same phenomenon with the KHV transmitter fish. The DNA band of the tested fish were about 290 bp, indicated these fishes were contaminated by the same KHV which transmitted by KHV transmitter fish during the cohabitation periods.

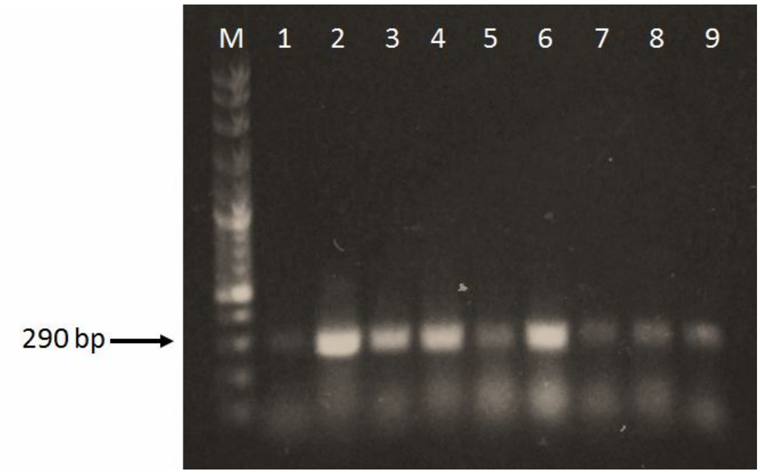

Figure 4. Molecular confirmation result for KHV infectious in the tested fish. 1: control (-) fish; 2-8: sample fish; 9: control (+) fish; M: marker.

Phenotypic performance. The phenotypic performance of fish after 90 days rearing period in net cage is presented in Table 3. The results showed that Wildan strain had the highest final body weight and biomass at harvest, while Rajadanu strain had the smallest features. Table 3 also showed the best strain in survival rate, that was Sinyonya strain. However, Sinyonya strain was not give a good result in biomass at harvest time due to bad performance of the strain in growth such as low final body weight and length.

Table 3

Phenotypic performance (±S.E.) of five strains of common carp which reared in net cage pond for 90 days

Strain	*Body length (mm)*	*Body weight (g)*	*Survival rate (%)*	*Biomass (kg)*
Rajadanu	94.6 ± 4.5^{ab}	28.8 ± 6.6^{b}	54.6 ± 4.6^{b}	8.7 ± 5.1^{a}
Sutisna	100.5 ± 2.9^{a}	40.0 ± 3.9^{a}	48.5 ± 6.9^{b}	10.7 ± 1.8^{a}
Majalaya	101.1 ± 1.6^{a}	37.6 ± 5.7^{a}	45.5 ± 1.8^{b}	9.4 ± 1.8^{a}
Wildan	99.6 ± 6.0^{a}	42.6 ± 2.8^{a}	54.6 ± 5.5^{b}	12.8 ± 6.4^{a}
Sinyonya	85.9 ± 4.1^{b}	29.3 ± 2.8^{b}	74.6 ± 5.5^{a}	12.0 ± 1.1^{a}

The significance values ($p > 0.05$) were showed by different superscript letters.

Discussion. The high number of MHC-II gene in Rajadanu strain may be happen due to the population that used in this study was selected previously in breeding program for disease-resistant of common carp in Research Institute for Fish Breeding (RIFB). This selection program was conducted using the same method, named MHC-II marker assisted selection. The Rajadanu strain which used in this research was the 3rd generation of Rajadanu strain cultured in RIFB since it was collected from Kuningan regency, West Java Province. Among five strains, Rajadanu was the most resistant to KHV, indicated by the highest degree of the survival rate in challenge test, up to 36.7%. It was followed by Majalaya, Sutisna and Sinyonya strains, accordingly, which corresponded to the percentage of individuals carrying MHC-II markers in those populations. The results indicated that common carp strains with high percentage of individual carrying MHC-II gene have a specific immune response associated with higher survival rate. Sucipto et al (2011) described that MHC-II gene was a specific defense system that plays role in eliminating parasites, bacteria and neutralizing viruses. In general, in the immune response to viral disease, viral peptide derivatives derived from cytosolic virus biosynthesis were presented by MHC-I molecules to be cytotoxic on $CD8^+$ T cells (Jia et al 2018). In the MHC II molecule, the viral peptide derivatives of degradation in the endosome/lysosome region were presented on $CD4^+$ T cells. The process would induce interferon production which was able to depress the spread of the virus and also stimulates the production of specific antibodies. Although only a minor fraction, these antibodies have direct activity as antivirals, where antibodies will help to control viral infections by activating complement system, facilitating phagocytosis and activating free antibodies from cytotoxicity cells. Thus, when the spread of herpesvirus-virion were reached final stage, and occurs inside the cell, viral peptides can enter the MHC-II system efficiently and presented to $CD4^+$ T cells. Several studies in humans and mice explained that $CD4^+$ T cells were important in controlling herpesvirus infection (Heller et al 2006; Wiertz et al 2007). Studies on MHV-68 rat herpesvirus showed that $CD4^+$ T cells were able to control infections in vivo (Christensen et al 1999). In addition, Landais et al (2004) and Heller et al (2006) reported that the presence of Epstein Barr (EBV) glycoprotein virus produced by the MHC-II molecule was able to kill virus directly by infecting B cells using $CD4^+$ T cells. In this study, it was suspected that the higher percentage of individu carrying MHC-II gene has an important role in increasing immune system in the fish by increasing the number of $CD4^+$ T cells. It affected fish ability to have a better survival rate compared to another fish strain with lower percentage of MHC-II gene.

Another strain with high percentage of MHC-II gene, namely Wildan strain, which reached a survival rate of 90% was also interesting to be learned. The Wildan strain used in this study was the fish which were directly collected from Kadupandak area, Cianjur regency, West Java Province. Naturally, Wildan strain has a high percentage of MHC-II gene indicated that this common carp population was high potential to be used for disease resistant breeding program of common carp. Nevertheless, the final result of the challenge test showed that Wildan strain was the lowest degree of survival rate than other strains. There are several reasons which can be explained as follows: (1) Analysis of percentage of MHC-II gene and challenge test of KHV resistance were conducted using different populations. MHC-II gene marker was analyzed in the parent of fish but the challenge test of KHV resistance was conducted using its progeny. Ariyanto et al (2015) reported that spawning with positive MHC-II in both of male and female broodstock has not always produced resistant progeny carrying MHC-II gene. Spawning with a positive MHC-II in male with a negative MHC-II of female and vice versa were produced progeny with different percentages of MHC-II gene. Spawning with negative MHC-II in both of male and female also had the opportunity to produce progeny with high percentage of MHC-II. The percentage of individual progeny carrying MHC-II gene was dependent on the heterozygote or homozygote in term of MHC-II of the parents. Based on those results, it is not yet certainly that the parent population with a high percentage of MHC-II will produce progeny with a high percentage of MHC-II, which that positively correlated to KHV resistance. Similarly, the parent population with a low percentage of MHC-II genes maybe produced the progeny with low or high resistance to KHV; (2) Another assumption affecting the final result of this challenge test was the mechanism of the MHC-II gene against the KHV attacks on each strain related to other characters such as growth and reproduction. Hayuningtyas et al (2015) reported that common carp population which had higher resistance to disease especially to KHV had a lower growth in pond. Other studies in several populations of common carp also showed similar results (Kirpichnikov et al 1993; Nielsen et al 2010; Bangera et al 2011). The same condition was suggested to occur in Wildan strain that had faster growth compared to other strains (Table 3), but had lower disease resistance (Table 2). The results of this study indicated that the resistance to disease especially to KHV in common carp was not only correlated with the presence of MHC-II gene but also had an impact on the decline in population growth rates.

Conclusions. Rajadanu and Wildan strains had the highest percentage of MHC-II genes, followed by Majalaya, Sutisna and Sinyonya strains. The results of the challenge test showed that the Rajadanu strain had the highest resistance to KHV, followed by Majalaya, Sutisna, Sinyonya and Wildan strains. The mechanism of resistance to KHV disease was not only absolutely determined by the high percentage of individual fish carrying MHC-II gene, but also related to the biological mechanisms and metabolism of other characters, such as the growth rate of the fish.

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