IDENTIFICATION OF MAJALAYA COMMON CARP STRAINS RESISTANT TO KHV INFECTION USING CYCA-DAB1*05 ALLELE AS THE MARKER

Alimuddin[*)#], Mubinun[**)], Ayi Santika[***)], Odang Carman[*)], Irvan Faizal[****)], and Komar Sumantadinata[*)]

[*)] *Department of Aquaculture, Bogor Agricultural University, Bogor 16680, Indonesia*
[**)] *Post Graduate Student of Aquaculture Science, Department of Aquaculture, Bogor Agricultural University. Present Address: Regional Freshwater Aquaculture Development Center, Jambi*
[***)] *Main Center of Freshwater Aquaculture Development, Sukabumi, West Java*
[****)] *Agency for The Assessment and Application of Technology, Jakarta*

(*Received 8 November 2010; Accepted 20 July 2011*)

ABSTRACT

The Cyca-DAB1*05 allele of major histocompatibility complex class II genes is recently suggested to have a link with the European common carp strain resistant to koi herpesvirus (KHV). In this study, a set of specific primers for Cyca-DAB1*05 was designed and applied as a marker to identify broodstocks of majalaya common carp strain subsequently used as a candidate resistant to KHV infection. From a total of 23 broodstock subjected to PCR analysis, two female and male fish, both having (P) and no Cyca-DAB1*05 (N), were selected and then diallelly mated. Disease resistance of progenies from 10 crosses was determined by a survival analysis in pond rearing and a laboratory challenge-test using cohabitation method. The results have revealed that the average survivals of PxP progenies for pond rearing and KHV challenge test were 86% and 100% higher ($P < 0.05$) respectively compared to that of NxN fish. Survival rate of PxN/NxP progenies was significantly lower ($P < 0.05$) than that of PxP fish. Furthermore, PCR analysis showed that almost 91% progenies of PxP crosses seemed to have a KHV resistant gene marker. Thus, this study suggests that the marker is associated with the KHV resistance in majalaya common carp strain, and farming of PxP progenies can be useful to increase common carp production.

KEYWORDS: Cyca-DAB1*05, molecular marker, koi herpesvirus, *Cyprinus carpio*

INTRODUCTION

Common carp (*Cyprinus carpio*) is one of the several most important aquaculture species in Indonesia. There are at least 6 common carp strains, namely majalaya, sinyonya, punten, rajadanu, cangkringan, and wildan (Sumantadinata, 1995; Aliah & Taniguchi, 1999). The majalaya is a major common carp strain farmed in Indonesia. Since 2002, common carp farming activity and its production level have decreased sharply because of koi or cyprinid herpesvirus (KHV/CyHV-3) infection. Several works had been performed to cope with KHV

Corresponding author: Laboratory of Fish Breeding and Genetics, Department of Aquaculture Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor 16680, Indonesia. Tel.: + 62 251 8622941
E-mail address: kmahardika@yahoo.com

infection in common carp, such as the use of phytomedicine (e.g. garlic extract and powder) and DNA vaccine (Nuryati *et al*., 2010). However, the wide application of those substances still has to be developed to meet the practical and economical means.

Improvement of broodstock and seed quality can be achieved by selective breeding program. However, the process requires more than five generations in order to obtain significant increase of genetic improvement. Recently, researchers have developed molecular marker-assisted selection (MAS) to accelerate the selection (Liu & Cordes, 2004) by avoiding unintended crosses (Martinez, 2007). The use of MAS is also potential as a fast track to increase genetic gain (Sonesson, 2007; Ibitoye & Idowu, 2010).

It is commonly found that several individuals have survived when KHV infected a majalaya common carp population. The survived fish may be genetically resistant to KHV as found in European common carp (Rakus *et al*., 2009). KHV-resistant European common carp is linked to Cyca-DAB1*05 allele of major histocomapatibility complex (MHC) class II (Rakus *et al*., ibid). Other linked molecular markers for KHV-resistant in European common carp have also been reported by Kongchum *et al*. (2010). Furthermore, the average survival of progenies from crosses between European common carp strains is higher than the purebred against KHV infection (Odegard *et al*., 2010). In this study, a specific primer set for Cyca-DAB1*05 as a marker was designed and then used in PCR amplification to select the broodstocks of majalaya common carp carrying the marker. The broodstock subsequently can be used for mass production of KHV-resistant progenies, and crossbreed with other strains.

MATERIALS AND METHODS

Primer Design and Cyca-DAB1*05 Allele Analysis

The nucleotide sequences of Cyca-DAB1*02 (GenBank: EU203666.1), Cyca-DAB2*02 (GenBank: EU203670.1), Cyca-DAB1*05 (GenBank: EU203669.1), and Cyca-DAB1*06 (GenBank: EU860997.1) were aligned using a software GENETYX ver. 7 to design a specific primer set for Cyca-DAB1*05 allele. The primer set is 5'-AATGGATACTACTGG-3', and 5'-TCGCTGACTGTCTGTT-3'. The size PCR product of Cyca-DAB1*05 is about 260 bp.

The majalaya common carp strain used in the study was the survived fourth generation after KHV infection and maintained in ponds at the Main Center of Freshwater Aquaculture Development, Sukabumi, Indonesia. The DNA genome of caudal fin from a total of 23 microchip-tagged fishes (13 females and 13 males) was isolated using a DNA Isolation Kit (Qiagen) according to the manufacturer's instructions. PCR analysis was performed in 20 µL of 10x *Ex Taq* buffer, 200 µM of dNTPs, 0.125 U of *Ex Taq* polymerase (Takara, Shiga, Japan), 2 µL of DNA as template, and 1 pmol of each primer. A total of 35 cycles of denaturation for 30 s at 94°C, annealing at 62°C for 30 s, and extension at 72°C for 30 s were performed. PCR was also performed for the β-actin gene as an internal control using this primer set: 5'-GTGCCCATCTACGAGGGTTA-3' and 5'-TTTGATGTCACGCACGATTT-3'. PCR amplification for the internal control was performed as follows: 5 min. at 94°C, followed by 30 cycles of 20 s at 94°C, 15 s at 68°C, 15 s at 72°C and 3 min. at 72°C. One microliter of the reaction was then electrophoretically separated using 0.7% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Progenies Production and Rearing

Four diallel crosses of Cyca-DAB1*05-carried fish (P x P) and non-carried fish (N x N), and two crosses for Cyca-DAB1*05-carried and non-carried fish (P x N) were performed to produce 10 families. Egg ovulation was induced by hormonal manipulation using ovaprim (Syndel Laboratories, Ltd.). Triplicate batches of fertilized eggs from each cross were incubated in 90-L glass aquaria at 28 ± 1°C. The *larvae were fed with Artemia nauplii* three times a day. Five-day-old larvae were transferred to a hapa (size 2 m x 2 m x 1 m) settled in a concrete pond of 300 m^2. Larval rearing density was 300 fishes per hapa. Fish were maintained for 60 days and fed with a commercial diet (protein content 28%) for three times daily. Thirty fishes per hapa were sampled every two weeks to measure the body weight. Survival rate was calculated at the end of fish rearing.

Ten fish samples from each cross were randomly selected after 45 days of rearing to determine the fish carrying Cyca-DAB1*05 allele. The DNA genome of caudal fin was isolated using a DNA Isolation Kit (Qiagen) according to the manufacturer's instructions.

KHV Challenge Test

Sixty-day-old KHV-free common carp (8-15 g of body weight) with a total of 30 fishes per family were adapted in 90-L aquaria at 21°C-23°C for three days before challenge test. The status of KHV-free was determined using PCR analysis as described by Gray *et al*. (2002). Challenge test was conducted using cohabitation method by adding three KHV-infected fish into each aquarium. Fish were reared for 30 days at 21°C-23°C, fed with a commercial diet (28% protein) for three times a day. Water quality condition was maintained by allowing water exchange for up to 50% every two days. The fish mortality was recorded everyday. Samples of five live and dead fish from each cross were collected to determine the presence of Cyca-DAB1*05 allele by using PCR analysis as described previously.

Statistical Analisis

Statistical analyses were performed by using the SPSS 11.0 microcomputer software package (SPSS, Chicago, IL, USA). Differences between crosses were determined by one-way ANOVA followed by Duncan's test. The level of significance was set at P = 0.05.

RESULTS

Broodstock Identification and Crosses

Among 26 (13 males and 13 females) common carp broodstocks identified by PCR method, 5 males (38.5%) and three females (23.1%) were revealed to have Cyca-DAB1*05 marker. Figure 1 shows an example of PCR product from female broodstock identification. The female fish no. 7 (chip no. 1828047551), no. 8 (chip no. 1828047832), and also no. 10 (chip no. 1828047812) were identified to have the marker. The male fish that have the marker were no. 3 (chip no. 1828047768), 6 (chip no. 1828085830), 9 (chip no. 1828085824), 10 (chip no. 1828085823), and 11 (chip no. 1828085829). In this study 2 males (fish no. 1828085824 and 1828085823) and two females (fish no. 1828047551 and 1828047832) carrying (P) the marker and the other two males and females fish carrying none (N) of the marker were chosen. The diallel crossing has resulted 8 families (P_1xP_1, P_1xP_2, P_2xP_1, P_2xP_2, N_1xN_1, N_1xN_2, N_2xN_1, N_2xN_2). The diallel cross of P_1xN_1 and N_1xP_1 was also generated.

Pond Survival and Growth Rate of Progenies

From 10 crosses (Figure 2), the average pond survival rates of PxP progenies (72.53± 2.66%) were higher ($P < 0.05$) than that of NxP/PxN (56.00 ± 3.17%) and NxN (38.97 ± 1.18%). There was no significant different in the pond survival rate of NxP, PxN, and NxN families, except for N_2xN_2 (37.87 ± 2.68%) and N_2xN_1 (11.00 ± 0.00%) is lower ($P < 0.05$) than P_1xN_1 (52.63 ± 3.21%), N_1xP_1 (59.30 ± 3.16%), N_1xN_1 (55.30 ± 2.65%), and N_1xN_2 (51.63 ± 2.71%). Highest number of progenies survived in pond

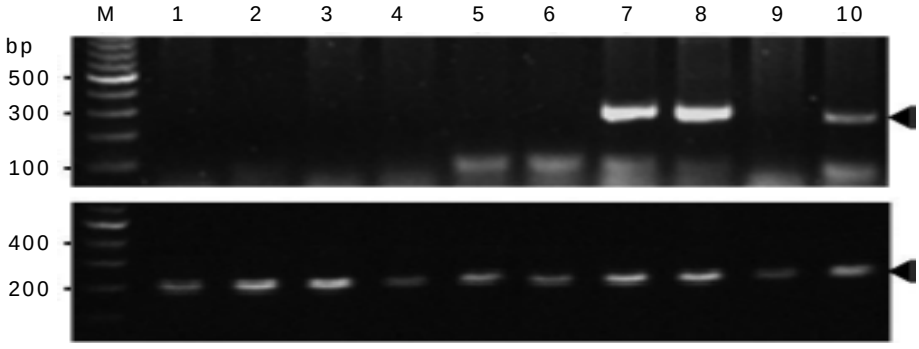

Figure 1. The results of screening for the presence of Cyca-DAB1*05 allele by PCR analysis with DNA templates extracted from caudal fin of broodstock common carp. A: PCR product using Cyca-DAB1*05 specific primers. Lanes 1 to 10, PCR product of broodstock individuals. M is 2-log ladder DNA marker (BioLabs, Inc., New England). The amplified fragment is about 260-bp in size (marked by arrow head). B: PCR product with b-actin gene primers [200-bp in size]

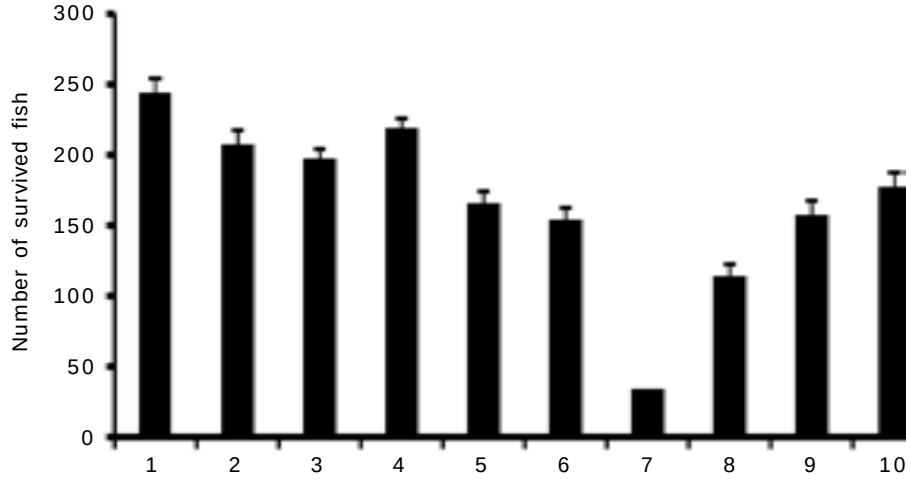

Figure 2. Number of survived fish after two months pond rearing. The X axis is progenies from (1) P_1xP_1; (2) P_1xP_2; (3) P_2xP_1; (4) P_2xP_2; (5) N_1xN_1; (6) N_1xN_2; (7) N_2xN_1; (8) N_2xN_2; (9) P_1xN_1; (10) N_1xP_1 crosses. A total of 300 fishes per hapa (2 m x 2 m x 1 m in size) was maintained in a concrete pond. Different letters on top the bars show statistically different ($P < 0.05$)

rearing was obtained in P_1xP_1 (81.73 ± 3.09%), followed by P_2xP_2 (73.20 ± 2.31%), P_1xP_2 (69.43 ± 3.50%), and P_2xP_1 (65.63 ± 2.37%). Moreover, the results of PCR analysis of the ten fish obtained randomly from each population showed that almost 91% PxP progenies have the marker compared to the PxN/NxP and NxN progenies with only 23.3% and 13.3%, respectively. Thus, a strong correlation between pond survival rate and the molecular marker has been observed.

The growth rate of PxP, NxN, and PxN progenies were similar ($P > 0.05$), except for N_2xN_1 ($P < 0.05$) (Figure 3). Furthermore, the growth of N_2xN_2 (1.075 ± 0.015 g/day) was slightly higher ($P < 0.05$) than that of P_1xP_1 (1.059 ± 0.006 g/day) and P_1xP_2 (1.061 ± 0.004 g/day). The data showed that due to lower rearing density on N_2xN_2, the survival of this progeny (37.87%) was lower ($P < 0,05$) than that of P_1xP_1 (81.73%) and P_1xP_2 (69.43%).

Challenge-Test Survival

After KHV challenge-test, the average survival rates of PxP progenies (78.61 ± 4.59%) were significantly higher ($P < 0.05$) than that of NxN (33.89 ± 2.93%), and NxP/PxN (11.67 ± 3.33%) (Figure 4). In addition, the survival rates of P_1xP_2 (94.4 ± 6.97%) and P_2xP_1 (85.53 ± 12.64%) progenies were slightly higher than that of PxP progenies (73.25 ± 4.60% of P_2xP_2, and 68.87 ± 8.36% of P_1xP_1). Thus, it is arguably concluded that both parents are contributed to the resistance of their progenies against KHV infection.

DISCUSSION

Marker assisted selection (MAS) has been suggested to be a useful tool for shortening the time of selective breeding to obtain high quality broodstocks. In this study, PCR method was applied with a specific primer set for Cyca-DAB1*05 allele as the MAS to identify the majalaya common carp broodstock capable of producing progenies resistant to KHV. As a result, challenge-test survivals of PxP progenies showed about two times higher compared to NxN progenies. This clearly indicates that there is a link between the molecular marker and survival of the progenies. Furthermore, the survival rates of PxN/NxP and NxN progenies were constantly lower than that of PxP when they were challenged in pond condition and cohabitated with KHV-infected fish. However, the survival rate of NxP/PxN fish in KHV challenge test was lower than that of in pond rearing. This suggests that other immunogenetic factors may take part in increasing pond survival of NxP/PxN progenies.

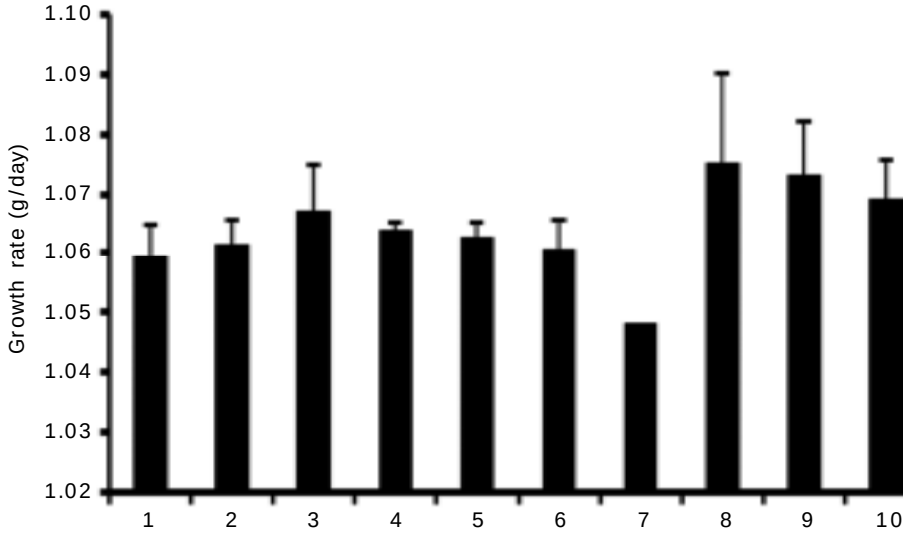

Figure 3. The growth rate (g/day) of fish after 2 months in the pond. The X axis is progenies from (1) P_1xP_1; (2) P_1xP_2; (3) P_2xP_1; (4) P_2xP_2; (5) N_1xN_1; (6) N_1xN_2; (7) N_2xN_1; (8) N_2xN_2; (9) P_1xN_1; (10) N_1xP_1 crosses. A total of 300 fishes per hapa (2 m x 2 m x 1 m in size) was maintained in a concrete pond. Different letters above the bars show statistically different ($P < 0.05$)

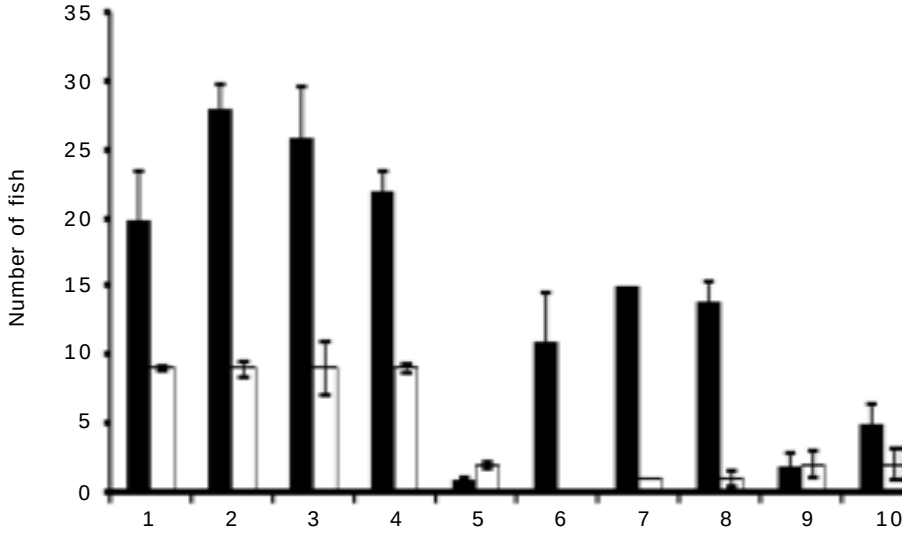

Figure 4. Number of survived fish after challenge test with KHV by cohabitation method (black bar) and number of fish having the molecular marker of Cyca-DAB1*05 allele (white bar). Three KHV-infected fish were cohabitated with 30 fishes from each cross for 30 days challenge test. DNA analysis was performed on ten fishes from each cross. The X axis is progenies from (1) P_1xP_1; (2) P_1xP_2; (3) P_2xP_1; (4) P_2xP_2; (5) N_1xN_1; (6) N_1xN_2; (7) N_2xN_1; (8) N_2xN_2; (9) P_1xN_1; (10) N_1xP_1 crosses. Different letters above the bars show statistically different ($P < 0.05$)

Variation of KHV resistance in terms of survival rate of progenies from different PxP cross was observed (Figure 2 and 4). In fact, about 91% progenies derived from those PxP carry the molecular marker. In addition, the results of PCR amplification of DNA from five dead fish after KHV challenge test showed that about 20% fish carried the marker. In this study, Cyca-DAB1*05 of MHC class II was used as the marker. In vertebrate including fish, immune response occupies the MHC class I and class II (Gillund *et al*., 2008). Thus, it is likely that MHC I may also be involved in immune regulation of common carp against KHV infection. Indeed, allele heterozigosity in Cyca-DAB1, e.g. *02/*05, *05/*05, and *05/*06, has been reported in European common carp (Rakus *et al*., 2009). This heterozigosity affects resistance of fish against KHV infection. Similar pattern may also exist in majalaya common carp strain, and this remains to be investigated in the future.

A similar size of PCR product of Cyca-DAB1*05 in PxP fish was obtained in several individuals of NxN progeny (Figure 3). Because of high homology of nucleotide sequences among Cyca-DAB1, specific primer set for Cyca-DAB1*05 was designed based on one and two nucleotide polymorphism. Changes in one nucleotide at 3' region of Cyca-DAB1 can allow annealing of the primer. Thus, it is more likely that DNA band of PCR amplification product from NxN fish is the result of annealing primer to the mutated nucleotide region of Cyca-DAB1.

As a consequence of variations in the resistance of common carp to KHV infection, fish breeders are required to select the suitable PxP crosses producing higher KHV-resistant seed. The seed from those crosses can directly be extended for nursery and grow-out as a shortcut way to increase common carp production. The use of selective breeding method to allow Cyca-DAB1*05 to be passed on from broodstocks to their progeny may also increase resistance of common carp to KHV infection. There are at least 6 common carp strains in Indonesia (Aliah *et al*., 1999). The use of Cyca-DAB1*05 marker to identify other common carp strains is also interested to be performed to provide various strains that are resistant to KHV. In addition, the survival of progenies from crosses of European common carp strains is higher than their purebred against KHV infection (Odegard *et al*., 2010). The KHV resistance of Indonesian common carp may also be improved by cross breeding program.

CONCLUSION

The molecular marker of Cyca-DAB1*05 allele can be used to identify individual broodstock of majalaya common carp strain producing progenies resistant to KHV infection. The progenies have high survival rates when it is cultured in ponds.

ACKNOWLEDGEMENT

The authors would like to thank Dwi Hany Yanti for her technical assistance in PCR analysis.

REFERENCES

Aliah, R.S. & Taniguchi, N. 1999. Comparison of genetic variability in nine domesticated stocks of Indonesian Common Carp using microsatellite DNA markers. *Fish Genetics and Breeding Science*, 28: 121-130.

Gillund, F., Dalmo, R., Tonheim, T.C., Seternes, T., & Myhr, A.I. 2008. DNA vaccination in aquaculture - Expert judgments of impacts on environment and fish health. *Aquaculture*, 284: 25-34.

Ibitoye, D.O. & Idowu, P.E. 2010. Marker-assisted-selection (MAS): a fast track to increase genetic gain in horticultural crop breeding. *African J. of Biotechnology*, 9: 8,889-8,895.

Kongchum, P., Palti, Y., Hallerman, E.M., Hulata, G., & David, L. 2010. SNP discovery and development of genetic markers for mapping innate immune response genes in common carp (*Cyprinus carpio*). *Fish & Shellfish Immunology*, 29: 356-361.

Liu, Z.J. & Cordes, J.F. 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, 238: 1-37.

Martinez, V. 2007. Marker-assisted selection in fish and shellfish schemes. In: *Marker-assisted selection: current status and future perspectives in crops, livestock, forestry, and fish* (Gumaraes, E., Ruane, J., Scherf, J., Sonnino, A., & Dargie, J. (Eds.). Food and Agricultural Organization (FAO) of the United Nations, Rome, Italy, p. 329-362.

Nuryati, S., Alimuddin, Sukenda, Soejoedono, R.D., Santika, A., Pasaribu, G.H. & Sumantadinata, K. 2010. Construction of a DNA vaccine using glycoprotein-25 and its expression towards increasing survival rate of KHV-infected common carp (*Cyprinus carpio*). *J. Natur Indonesia*, 13(1): 47-52.

Odegard, J., Olesen, I., Dixon, P., Jeney, Z., Nielsen, H.-M., Way, K., Joiner, C., Jeney, G., Ardó, L., Rónyai, A., & Gjerde, B. 2010. Genetic analysis of common carp (*Cyprinus carpio*) strains. II: Resistance to koi herpesvirus and *Aeromonas hydrophila* and their relationship with pond survival. *Aquaculture*, 304: 7-13.

Rakus, K.L., Wiegertjes, G.F., Adamek, M., Siwicki, A.K., Lepa, A., & Irnazarow, I. 2009. Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism. *Fish & Shellfish Immunology*, 26: 737-743.

Sonesson, A.K. 2007. Possibilities for marker-assisted selection in aquaculture breeding schemes. In: *Marker-assisted selection: current status and future perspectives in crops, livestock, forestry, and fish* (Gumaraes, E., Ruane, J., Scherf, J., Sonnino, A., & Dargie, J. (Eds.). *Food and Agricultural Organization (FAO) of the United Nations*, Rome, Italy, p. 310-328.

Sumantadinata, K. 1995. Present state of common carp (*Cyprinus carpio* L.) stocks in Indonesia. *Aquaculture*, 129: 205-209.

White, B.L., Schofield, P.J., Poulos, B.T., & Lightner, D.V. 2002. A laboratory challenge method for estimating *Taura Syndrome Virus* resistance in selected lines of Pacific white shrimp *Litopenaeus vannamei*. *J. of the World Aquaculture Society*, 33: 341-348.