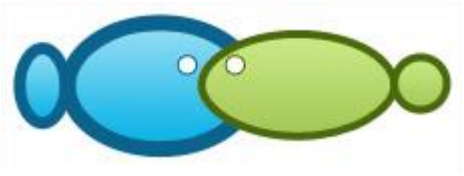

Development of a PCR marker for the identification of resistance to Motile Aeromonad Septicemia disease in African catfish (*Clarias gariepinus*)

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Abstract. Motile Aeromonad Septicemia (MAS) syndrome is caused by the bacteria *Aeromonas hydrophyla*, being the major disease hampering the production of African catfish (*Clarias gariepinus*) in early life stages for grow-out rearing. In addition to standard prophylactic treatments and curative measures, improving the natural and internal immunity through breeding programs is considered to be a prospective approach. While conventional breeding programs have been successful, marker-based selections provide alternative approaches. This study aimed to develop a molecular marker along with its primer set that is capable of discriminating individuals having differential resistance against *A. hydrophyla* infection. 300 juvenile African catfish of 8 cm average total length were used in the experiment. For the challenge test, the fish were intramuscularly injected with *A. hydrophyla* culture (10^8 CFU), and monitored for 7 days. Following the challenge test, the genomic DNA of the surviving and dead fish was extracted as template for the PCR reaction. 20 primer sets were designed based on Major Histocompatibility Complex (MHC I) alleles deposited in the Genebank and were used in PCR reactions to screen for the presence of MHC I allele within individuals both alive and dead. Results showed that an amplicon of 1000 bp amplified by one of the primer set was present uniquely in the surviving fish. Further analysis by sequencing suggested that this amplicon had a high similarity to an allele Clg-UAA*07 deposited in the gene bank. In summary, a primer set capable of amplifying a single band amplicon that can discriminate between the resistant and vulnerable *C. gariepinus* juveniles has successfully been obtained. It can be used to detect and select individual African catfish resistant to *A. hydrophyla* infection. Further studies in terms of its inheritance are required to be able to use it in a breeding program.

Key Words: *Aeromonas hydrophyla*, disease resistance, genetic marker, Major Histocompatibility Complex.

Introduction. The African catfish (*Clarias gariepinus*) has been an important and a major freshwater cultured species in Indonesia. In 2017, its production reached 1.126 million tons, which placed it as the second biggest production among ten major farmed fishes (KKP 2018). It serves mainly as a product that supports the food security of Indonesian people. While the demand for this species has been steadily increasing, efforts to boost grow-out production have often been hampered by the lack of continuous supply of quality seed resulting from disease in fingerling production. In freshwater systems of tropical environments, the Motile Aeromonad Septicemia (MAS) are the most frequently encountered disease outbreaks (Karunasagar et al 2003; Nielsen et al 2001), caused by the pathogenic bacteria *Aeromonas hydrophyla* (Suprapto et al 2017). Experimental evaluation with African catfish showed that the infected populations may suffer 20 to 90% mortality (Anyanwu et al 2015), incurring substantial losses to the aquaculture industry (Peterman & Posadas 2019; Sharma et al 2008).

The common measures to cope with this disease in aquaculture have been the implementation of different types of drugs and chemicals for either prophylactic or curative purposes (Singh & Singh 2018), by stimulating the external or inducible

immunity of fish. These include the use of chemotherapeutic drugs, phytobiotics (Cristea et al 2012; Nafiqoh et al 2019; Sharma et al 2008), probiotics (Al-Dohail et al 2009; Al-Dohail et al 2011), and vaccines (Sukenda et al 2017). Comprehensive reviews on the management of this disease have been discussed by Harikrisnan & Balasundaram (2005) and Stratev & Odeye (2017). Although the abovementioned measures have been capable of managing the disease, they also present some disadvantages. Antibiotics and other chemicals may leave residue within the fish body, may potentially pollute the environment, and may increase the resistance of pathogenic agents toward the chemicals (Poonsawat et al 2009). The vaccination, while it may be more suitable for food safety and environmental purposes, may not be economically feasible to the farmers, particularly for freshwater fish in which the profit margin is an issue. Therefore, apart from the curative measures applying a variety of chemical and biological agents (Singh & Singh 2018), efforts have been made towards improving the internal immunity of fish by establishing genetically more resistant or tolerant fish strains through breeding programs (Fjalestad et al 1993).

To date, conventional breeding programs such as selective breeding and crossbreeding have dominated the techniques of producing genetically improved fish breeds for aquaculture (Hulata 2001). The GIFT strain of tilapia, (*Oreochromis niloticus*) (Eknath et al 1998), salmon (*Salmo salar*) (Quinton et al 2005), and the Mutiara strain of African catfish (Iswanto et al 2016) are a few of established quality fish breeds produced by conventional breeding programs. In Indonesia, up to 2019, around 90% of 34 fish strains released for aquaculture were produced by conventional breeding programs, namely selective breeding or crossbreeding, while the remaining three breeds were produced by incorporating molecular markers. The former techniques dealt with growth related traits, while the latter targeted the improvement of disease resistance.

Although conventional breeding programs such as selective breeding have been proven to be effective in producing improved strains of economically important traits, particularly growth related traits, they have inherent disadvantages. In general, a conventional selective breeding technique requires a number of generations of selection in order for a significant genetic gain to be observed. Depending on the interval generation of species, this may take many years. In addition to the relatively longer time required, these techniques are also less suitable for traits that are difficult or costly to measure in live candidates, such as disease resistance, flesh quality, feed conversion efficiency and salinity or temperature tolerance (Sonesson 2007), or for traits expressed late in life (Haldar 2018). Marker-based breeding programs that work by exploiting the relationship between phenotypic traits of interest and molecular markers underlying those traits have features that allow addressing these limitations (Sonesson 2007).

The prerequisite for the implementation of marker-based breeding program is the availability of markers that associate with the traits of interest. One of the candidate genes that could be used as a marker for resistance to pathogens is the MHC class I. The MHC genes are candidate genes that play a role in innate and adaptive immune responses. The main advantage of the MHC class I molecules as genetic markers is the diversity in alleles that are suspected to be associated with disease resistance. Several studies exploring the presence of the markers and their utilization in breeding program have been conducted with salmon, (Wynne et al 2007), common carp (*Cyprinus carpio*) (Alimuddin et al 2011; Rakus et al 2009; Syahputra et al 2016), tilapia (Poonsawat et al 2009) and African catfish (Azis et al 2015b; Suprapto et al 2017). Regarding MAS disease in African catfish, a study with the Sangkuriang strain of African catfish found that three different DNA fragments, namely 300, 500 and 1000 bp in size were potentially linked to the resistance or susceptibility to *A. hydrophyla* infection (Azis et al 2015b). They showed that the PCR products have a high similarity to MHC I allele 9 and 17 in African catfish. However, the multiple bands of MHC alleles resulting from this study left several questions. For instance, which DNA fragment out of the three bands is really associated with the phenotype? From a practical point of view, the number of bands make them less practical for the purpose of identification. The current study was designed to address those unresolved questions. Specifically, it aims to determine a single and specific DNA marker of *C. gariepinus* associated with resistance to *A. hydrophyla* infection.

Material and Method

Experimental design. The study was conducted from May to September 2016, at the research facilities of the Research Institute for Fish Breeding (RIFB), Subang, West Java Province, Indonesia. Two groups of experimental fish, namely *A. hydrophyla* injected and non injected fish were prepared. While the former group was used for both determination of LD_{50} and challenge test, the latter group was used as a control. A more detailed description on the implementation of the experiment is described in the following sections.

Bacterial isolate of A. hydrophila. Bacterial pathogen *A. hydrophila* isolates code number AH-3 was provided by the Installation of Research and Development of Fish Diseases, Depok, Indonesia. The isolate was rejuvenated by sub-culture on TSB (*Tripticase Soy Broth*) medium. Before being used, the isolate was further incubated at 28^{o}C for 24 hours.

Fish material. The fish used in this study were juveniles ranging from 7 to 9 cm in size. They were offspring originated from the spawning of 13 spawning pairs. The fish were Mutiara strain of African catfish that was launched as new strain for aquaculture by the Indonesia's Ministry of Marine Affairs and Fisheries in 2015. The strain was developed from a synthetic base population, which combined four African catfish populations already existing in Indonesia through mass selection for growth improvement (Imron et al 2015). Specifically, the fish were the third generation (G-3) of the African catfish breeding program for fast growth conducted at the RIFB.

Determination of LD_{50} and challenge test with A. hydrophila. Before performing a challenge test, the dose of pathogenic bacteria *A. hydrophyla* causing 50% fish morality (LD_{50}) was determined. This was done by intramuscularly injecting each of the 30 test fish with 0.1 mL of *A. hydrophila* culture containing 10^6-10^8 CFU mL^{-1}. The fish were raised in an aquarium of 60x40x40 cm, equipped with aeration. This treatment was done in three replicates. As a control, we used juveniles without injection of *A. hydrophila*, which were raised in the same condition as the treated fish. An hourly observation was carried out to record fish mortality rate until 50% of the tested fish died. The calculation of LD_{50} dose followed recommendation by Reed & Munch (1938). The LD_{50} dose resulting from this step was then used to perform a challenge test.

The challenge test involved 300 healthy juvenile African catfish of similar size range to those used in the LD_{50} test. They were stocked in 10 glass aquaria of 60x40x40 cm filled with 36 L of water. Each aquarium was equipped with an aeration system and was stocked with 30 fish. Prior to starting the test, the juveniles were allowed to acclimatize to the new environment for two days. An intramuscular injection using LD_{50} dose that had previously been obtained was applied to each individual fish. The challenge test was carried out for 7 days. During the course of the challenge test water quality in each aquarium was maintained in good condition by keeping the aeration system working continuously and 50% water replacement was carried out every two days. An hourly observation focusing on mortality was made following the injection with *A. hydrophyla*. The dead fish during the course of the challenge test were immediately removed and preserved for clinical observations and the tissue was incised for genomic DNA extraction.

Primer design of MHC I alleles. To identify whether an individual fish carried MHC alleles, sets of primers were designed using information about MHC I alleles of *C. gariepinus* already existing in the Genebank (Wachirachaikarn et al 2009). A total of 21 MHC I allele sequences were downloaded and specific primer sets for each of these alleles were designed. Although the size of MHC I allele sequences deposited in the Genebank ranged from 543 to 549 base pairs, the primers, which were designed using PrimerBLAST, were set to amplify only a partial length of them ranging from 240 to 298 bp. The list of gene accession numbers, primer sequences and the expected size of the amplified product are presented in Table 1.

Table 1

Accession number, primer sequences and the expected product

No	*Accession numbers*	*Primer sequences*		*TA (°C)*	*Size of PCR product (bp)*
1	EU714302	01-154 F 01-401R	TGTGGGTAATCTGATGGGTCG CCAGGTAGTTCTTCCTGCCC	57	248 bp
2	EU714303	02-155 F 02-398 R	GTGGGTAATCTGATGGGTCGT GGTAGTTCTTCCTGCCCACA	57	244 bp
3	EU714304	03-156 F 03-400 R	TGGGTAATCTGATGGGTCGT CAGGTAGTTCTTCCTGCCCA	57	245 bp
4	EU714305	04-155 F 04-399 R	GTGGGTAATCTGATGGGTCGTT AGGTAGTTCTTCCTGCCCAC	58	245 bp
5	EU714306	05-153 F 05-400 R	ATGTGGGTAATCTGATGGGTCG CAGGTAGTTCTTCCTGCCCAC	58	248 bp
6	EU714307	06-155 F 06-397 R	GTGGGTAATCTGATGGGTCGT GTAGCTCTTCCTGCCCACAG	58	243 bp
7	EU714308	07-123 F 07-408 R	TGCAGAGTACTCAGGAGACC TCTTTCTCCAGGTAGTTCTTTCGAT	57	285 bp
8	EU714309	08-140 F 08-418 R	CATGCAGGGTCAGGAGGAGA CTCGATACACTCTTTCTCCAGGT	58	298 bp
9	EU714310	09-161 F 09-418 R	ATGGGTACACTGATGAGCCG ACACTCCTTCTCCAGGTAGTTC	57	258 bp
10	EU714311	10-155 F 10-420 R	ATGGGTACACTGATGGGTCGT CACTCGATACAGGTGTTCTCATAA	58	266 bp
11	EU714312	10-155 F 10-409 R	ATGGGTACACTGATGGGTCG GGTGGTCTCATAATAGTTCTTCAGG	57	255 bp
12	EU714313	11-157 F 11-420 R	GGGTACACTGATGGGTCGTTT CACTCGATACAGGTGTTCTCATA	58	264 bp
13	EU714314	12-133 F 12-412 R	TCAGGAGTGGTTCAAAGCCAATA ACACTCGTTCTCCAGGTAGTTCT	58	280 bp
14	EU714315	13-146 F 13-395 R	AAAGCCAGTGTGGGTATAGTGA CGTATCCCTTCCAGTAATCAGCA	57	250 bp
15	EU714316	14-121 F 14-396 R	ACAGCAGGGTACTGAGGAGA TATCCCTTCAGGTAATCATTCCTTC	58	276 bp
16	EU714317	15-154 F 15-397 R	TGTGGGTATAGTGATGGAGCG GTAGTCCTCCCTGCGCTTAT	57	244 bp
17	EU714318	16-156 F 16-395 R	TGGGTAATCTGATGCAGCGT AGTCCTCCCTGCGCTTATTAAA	58	240 bp
18	EU714319	17-158 F 17-410 R	GGTGTGGGTATACTGATGGGTC TCAGGTAGTCCTCTGTTCCCTT	57	253 bp
19	EU714320	18-127 F 18-419 R	TGCACGGGTTGAAGAACAGA TGCAGGTGTTCTCCAGGTAG	58	293 bp
20	EU714321	19-143 F 19-418 R	CAGAGCTTCAAGGCCAGTGT GCAGGTGTTCTCCAGGTAGT	58	276 bp
21	EU714322	20-139 F 20-420 R	AGAACAGAGCTTCAAGGCCA ATGCAGGTGTTCTCCAGGTAG	58	282 bp

Note: TA - annealing temperature.

DNA extraction. Genomic DNA of each sample from surviving and dead fish after the challenge test was extracted using a DNA extraction kit, following the protocols recommended by the manufacturer (GeneJet Genomic DNA Purification, Thermo Scientific). MHC I gene detection was performed for both surviving and dead juveniles following the challenge test. Briefly, the protocol for DNA extraction consists of a series of steps including cell lysis, DNA precipitation, binding DNA to the column, washing, and elution. Cell lysis was performed by weighing approximately 10 mg of tissue sample and mincing it with a surgical blade into small pieces. The tissue was lysed by adding 180 µL of lysis digestion buffer, 20 µL of proteinase K, followed by incubation at 56°C for 2 hours, the addition 20 µL of RNase A solution, and incubation at room temperature for 10 minutes. 200 µL of lysis solution was added and it was vortexed for 15 seconds. The lysate DNA was then precipitated by adding 400 µL of 50% ethanol. Binding DNA to the column was carried out by centrifuging the mixture lysate DNA and ethanol at 6000 xg

for 1 min. The column binding DNA was then washed twice by adding 500 µL of washing buffer 1 and washing buffer 2, followed by centrifugation at 8000 xg for 3 minutes and 12000 xg for 3 minutes, respectively. Finally, the DNA bound to the column was eluted by adding 200 µL elution buffer, incubating it for 2 minutes in room temperature and terminated by centrifuging it at 8000 xg for 1 minute. To check the success of the genomic DNA extraction process, the sample was run in mini horizontal gel electrophoresis. The sample was loaded in 1.5% (w/v) agarose gel, powered with 100 volt electricity and run for 35 minutes. The gel was stained with Peq Green (0.5 µg mL^{-1}) and viewed using gel documentation system (ultraviolet transillumination).

Screening for PCR primers of discriminatory prospect. To obtain PCR primer sets capable of amplifying amplicons suitable to be molecular markers, the 21 primer sets that have been designed were run in PCR reaction using genomic DNA template extracted from the surviving or dead fish, as previously described. For this purpose, every single primer set was run on a subsample of 20 surviving and dead fish, respectively. The parameters of interest included amplifying capacity (1), patterns of amplicon (2), estimated size (3) and uniqueness (4). The amplifying capacity refers to the capacity of the primer to produce amplicons in the PCR reaction, while the patterns of amplicons refer to the number of bands resulting from an individual sample, whether they came in a single or multiple bands. The estimated size refers to the sizes in base pair of amplicons. The uniqueness refers to the presence of the band in relation to resistance, namely whether their presence was specific to either surviving or dead fish, or they were present in both groups. The prospective MHC I alleles, namely those that of single band, single size and unique, were sequenced to check for their identity against the listed MHC I alleles. The DNA sequencing was carried out at 1st Base Sequencing INT-Singapore using A30 XL BI PRISM 3730 XL Genetic Analyzer (Applied Bio system, USA).

Screening for specific MHC 1 alleles based on the selected primer. Based on the results of previous steps, the amplification of a prospective MHC I allele, namely that capable of discriminating between the surviving and dead fish, was performed using the primer set 07-123-F (5'- TGC AGA GTA CTC AGG AGA CC -3') and 07-408-R (5'- TCT TTC TCC TTT CGA T -3') in a thermal cycling system (Mycycler, Biorad). PCR standard was performed in a final volume of 25 µL, using a commercial kit master mix Maxima hot-start PCR master (Thermo Scientific) for 35 cycles with the following PCR conditions: denaturing at 94^{o}C for 30 s, annealing at 59^{o}C for 30 s, and extension at 72^{o}C for 1 min. To ensure the quality of the genomic DNA template, the amplification of the same samples using a specific primer β-actin: bact-F (5'- AT GAA GGT TAT GCT CTG CCC -3') and bact-R (5'- CAT ACC CAG GAA AGA TGG CTG -3') designed from a catfish growth hormone sequence (Accession JF303887.1) as an internal control was carried out. The expected size of β gene-actine was 300 bp. The PCR reaction implied incubation at 94^{o}C for 30 s, annealing at 55^{o}C for 30 s, and extension at 72^{o}C for 1 min for 30 cycles. The PCR products were separated on 1.5% (w/v) agarose gel, stained with Peq Green (0.5 µg mL^{-1}) and visualized under an ultraviolet transillumination system.

Data analyses. PCR primer sets were classified as having discriminatory power when their amplification products were single banded, single sized, and unique, meaning that their presence was limited to either surviving or dead fish only. The first two criteria were analysed descriptively, while the last criterion was statistically tested using t-test at alpha 0.05, by Micorsoft Excel software. To ensure the identity of the prospective marker, the DNA sequences resulting from amplification using the prospective primer set were aligned against those in the gene bank database using BLAST (https://www.ncbi.nlm.nih.gov/).

Results and Discussion

LD_{50} and A. hydrophila challenge tests. The result of the LD_{50} test showed that the survival rate of fish from each treatment was different in time (Figure 1). The higest mortality occurred in fish injected with 10^9 CFU mL^{-1} concentration of *A. hydrophila*

(87.0%±3.61%). Futhermore, mortalities successively decreased for fish injected with 10^8 CFU mL^{-1} bacteria (50.0%±0.58%), 10^7 CFU mL^{-1} (25.0%±2.08%), and 10^6 CFU mL^{-1} (3.0±0.8%). These results show that the LD_{50} of *A. hydrophila* bacteria in this study was 10^8 CFU mL^{-1}. This LD_{50} dose was subsequently used to screen individuals that were resistant from those that were susceptible.

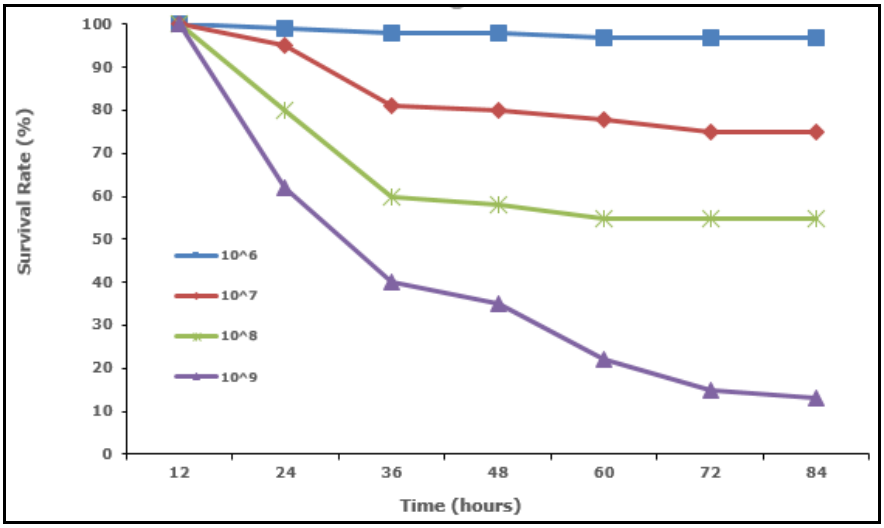

Figure 1. Survival rate (%) of African catfish (*Clarias gariepinus*) juveniles following the injection with *A. hydrophila* (CFU mL^{-1}) in determination of LD_{50}.

Specific MHC I markers and primers. The summary of a series of PCR analyses using 21 primer sets on both surviving and dead fish following the challenge test is presented in Table 2. The table shows several important features. First, all 21 primer sets successfully amplify PCR products. This means that the design of primer sets matched with the DNA binding site in the genome and PCR conditions were conducive for DNA fragment amplification. Secondly, despite primer ability to produce amplicons, the features of the amplicons were not in concordance with those expected. For instance, although all the primer sets were designed to produce a single band, several primer sets resulted in double or even multiple bands. The lack of concordance was also observed in terms of size, the sizes of several bands being beyond the expected ranges. Finally, the most interesting result in Table 2 is the presence of a primer set with an amplification product that met the criteria of having discriminatory features.

This primer set, namely primer 07-123F and 07-408R produced a single amplicon of 1000 bp. Additionally, and most importantly, it was also unique, as it was amplified in surviving fish only, being nearly absent in the dead fish (Table 2 and Figure 2A). Indeed, one dead fish was detected to carry the band. However, a careful evaluation to identify the causes of the death found that it was not caused by *A. hydrophyla* infection. The absence of this amplicon in the dead fish was believed to be caused by the fact that the dead fish did not carry the same allele as the surviving fish. This was supported by the presence of β-actine band as a positive control, which was proven to be present in both the surviving and dead fish (Figure 2B). The other supporting evidence came from the control population, namely those that were not injected with *A. hydrophyla*. Within this population, the 1000 bp amplicon was variably present. It occurred in 7 out of 13 tested fish, meaning that the control population consists of individuals that are carriers and non-carriers of MHC I allele (Figure 2C). Therefore, it can be concluded that the band was the expected product. However, the issue of uncertainty remained as the size of the amplicon was far beyond what was expected. As it can be seen in Table 1, the expected amplicon size based on the designed primer was 285 bp instead of 1000 bp.

Table2

Summary of screening for specific DNA fragment and its corresponding primer set that amplified it

No	Primer identity	AA (Y/N)	Size conf (Y/N)	Banding patterns (S/D/M)	Uniqueness (L/D/B)	Number of amplicon		t test
						Dead (n=20)	Live (n=20)	
1	01-154F & 01-401R	Y	N	D/M	B	9	7	ns
2	02-155F & 02-398R	Y	N	D	B	20	20	ns
3	03-156F & 03-400R	Y	N	S/D	B	4	4	ns
4	04-155F & 04-399R	Y	N	D	B	18	19	ns
5	05-153F & 05-400R	Y	N	D/M	B	20	20	ns
6	06-155F & 06-397R	Y	N	S/D/M	B	20	20	ns
7	07-123F & 07-408R	Y	N	S	L	1	20	*
8	08-140F & 08-418R	Y	N	D	B	17	19	ns
9	09-161F & 09-418R	Y	N	D	L	16	20	ns
10	10-155F & 10-420R	Y	N	D/M	B	20	20	ns
11	11-155F & 11-409R	Y	N	S/D/M	B	19	20	ns
12	12-157F & 12-420R	Y	N	D	B	2	2	ns
13	13-133F & 13-412R	Y	N	D/M	B	17	20	ns
14	14-146F & 14-395R	Y	N	D/M	B	18	20	ns
15	15-121F & 15-396R	Y	N	S/M	B	20	20	ns
16	16-154F & 16-397R	Y	N	S/D	B	20	20	ns
17	17-156F & 17-395R	Y	N	D	B	3	3	ns
18	18-158F & 18-410R	Y	N	D	B	5	7	ns
19	19-127F & 19-419R	Y	N	S/D	B	20	20	ns
20	20-143F & 20-418R	Y	N	D/M	B	20	20	ns
21	21-139F & 21-420R	Y	N	S/D/M	B	4	3	ns

Note: AA refers to the amplifying ability, with Y for yes and N for no, size conformation indicates whether amplicon sizes are in concordance with the expected size; pattern of PCR product refers to the status of the amplicon, which could be single (S) double (D) or multiple (3 or more) bands; uniqueness refers to whether the band presence is in Live (L), Dead (D) or both (B) fish; the asterisk and ns in the t test column refer to statistically significant differences ($P<0.05$), and not significant, respectively.

Sequence analysis of the marker candidate. BLAST analysis results indicated that the specific PCR products of surviving African catfish had a high identity with the MHC I genes from African catfish (Table 3). The alignment of the 1000 bp amplicon sequence amplified by the primer set 07-123F and 07-408R shows that it had the highest identity to Clga-UAA*7 and Clga-UAA*8 alleles. With a query cover of 83% and total score of 326, the identity to both allele was 99%. Identities to the other ten MHC I alleles were lower, ranging from 80 to 87%. Additionally, query cover and total score for these alleles were also lower, ranging between 77 and 81% and 121 and 202, respectively. The identity of the prospective marker to the remaining 8 alleles did not exist, meaning that the 1000 bp prospective marker resulting from this study was not part of these alleles (Table 3).

Condition of LD_{50} test. The LD_{50} dose found in this study was slighty higher than that found by Aziz et al (2015b) (10^6 CFU mL^{-1}) or the one found by Suprapto et al (2017) (10^5 CFU mL^{-1}). It is of interest to note that the present study, as well as the previous studies by Azis et al (2015b) and Suprapto et al (2017), used the same source of *A. hydrophyla* isolates. They were obtained from the Research Institute for Freshwater Aquaculture, Bogor. While Azis et al (2015b) used isolate code number AHL 110306-3, the present study used the isolate code number AH-3, which is the same isolate as that used by Suprapto et al (2017). The presence of variation in the LD_{50} dose between one LD_{50} test and another, despite using the same source of *A. hydrophyla* isolates, might be caused by differences in the level of bacterial virulence, level of fish population resistance and environmental conditions during the tests (Song et al 2014). Due to these

conditions, a determination of LD_{50} dose is needed before any challenge test against particular pathogens is performed.

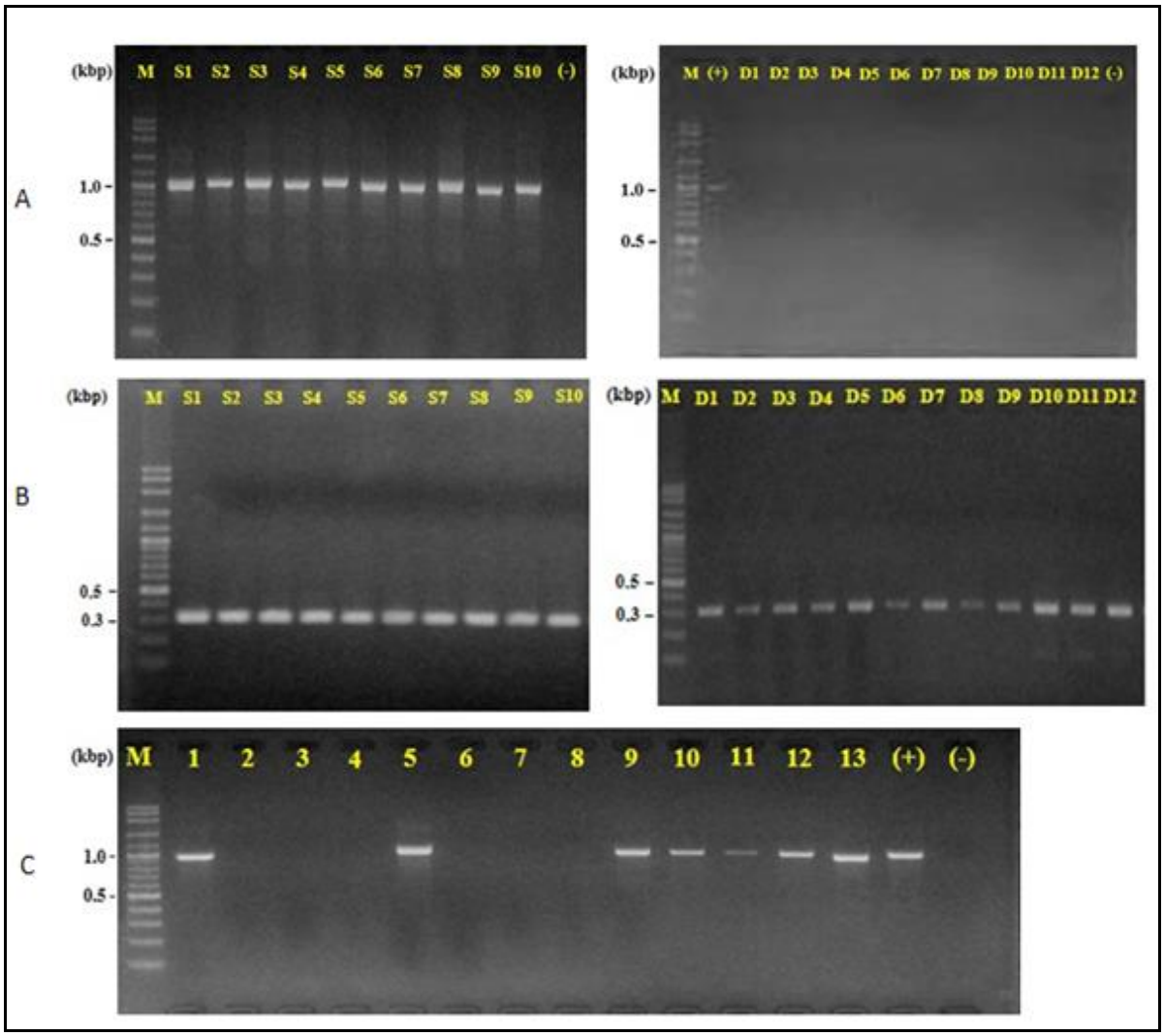

Figure 2. Detection of major histocompatibility complex I as molecular marker in Mutiara strain of African catfish (*Clarias gariepinus*). An amplicon of 1000 bp was present in the surviving fish (S1-S10) and was absent in dead fish (D1-D12), following the challenge test (A); a β-actin band of 300 bp as internal control of genomic DNA was present in both the surviving and dead fish (B); the 1000 bp amplicon was variably present in fish without injection (C). M - marker of DNA fragment size (100-3000 bp, Vivantis), (+) positive control and (-) negative control.

Characteristics of the prospective marker. The most easily noticeable features of amplicons resulting from the screening of the primer sets were the discrepancies between what was produced and what was expected. The expected amplicons, according to the primer sets that have been designed, would be those with a single band of expected size. However, as can be seen in Table 2, with the exception of primer 07-123F and 07-408R, all the other primer sets resulted in amplicons of combined banding patterns. This meant that in a group, namely the surviving or the dead fish, individuals might show different banding patterns after being run on PCR with the same set of primers. While a set PCR conditions may contribute to this, the most plausible explanation for this situation was laid on the PCR template being used. All the primers were designed based on the sequence of messenger RNA (mRNA), which were transcribed into cDNA through reverse transcriptase reaction. Through this process, any noncoding DNA spanned in between would be removed, so that only the coding sequences would be transcribed into cDNA.

The expected size of the products were estimated based on an assumption that no noncoding DNA spanned between the gene or DNA fragment to which the primer sequence attached to, which in this case did not hold. In the present study, it was genomic DNA, instead of mRNA, that was used as a template in the PCR reaction. Consequently, any noncoding DNA present between primer binding sites was also amplified leading to an additional length of DNA, in addition to the one expected. Despite of size discrepancy, sequence alignment of prospective markers against Genebank database confirmed that it represented the MHC I allele 7. All the MHC I allele, including the MHC I allele 7, as shown in the Genebank database, represent a UAA type (Wachirachaikarn et al 2009).

There have not been comprehensive reports exploring the MHC genes in the genus *Clarias,* particularly for *C. gariepinus*. Hence the descriptions of Clga-UAA*07 allele presented were deduced from that of the Medaka and Zebra fish, as they reside within the same clade as catfish (Grimholt 2016). In teleost fish, there are five lineages of MHC genes, namely lineages U, Z, S, L and P (Grimholt 2016; Grimholt et al 2015). The UAA type is a variant of the U lineage that is representative of classical loci, contains a wide range of allele and is expressed in most tissues (Grimholt 2016). In *C. gariepinus*, 21 variant alleles MHC I have been reported (Wachirachaikarn et al 2009), while in salmon and rainbow trout (*Oncorhynchus mykiss),* 48 variant alleles have been reported (Maccari et al 2017).

Table 3

Summary of BLAST parameters of the prospective 1000 bp amplicon against 21 MHC 1 alleles deposited in the Genebank

No	*Description*	*Total score*	*Query cover*	*E value*	*Identity*	*Accession*	*Remarks*
1	Clga-UAA*07 allele	326	83%	7.00E-94	98.91%	EU714308.1	
2	Clga-UAA*08 allele	326	83%	7.00E-94	98.91%	EU714309.1	
3	Clga-UAA*18 allele	202	81%	1.00E-56	87.22%	EU714319.1	
4	Clga-UAA*19 allele	202	81%	1.00E-56	87.22%	EU714320.1	
5	Clga-UAA*20 allele	202	81%	1.00E-56	87.22%	EU714321.1	
6	Clga-UAA*12 allele	152	77%	1.00E-41	83.04%	EU714313.1	
7	Clga-UAA*10 allele	152	77%	1.00E-41	82.94%	EU714311.1	
8	Clga-UAA*11 allele	152	77%	1.00E-41	82.94%	EU714312.1	
9	Clga-UAA*14 allele	135	77%	1.00E-36	81.07%	EU714315.1	
10	Clga-UAA*09 allele	124	77%	3.00E-33	79.88%	EU714310.1	
11	Clga-UAA*16 allele	128	80%	2.00E-34	79.78%	EU714317.1	
12	Clga-UAA*17 allele	121	77%	4.00E-32	79.77%	EU714318.1	
13	Clga-UAA*01allele	0	0	0	0	EU714302.1	NS
14	Clga-UAA*02 allele	0	0	0	0	EU714303.1	NS
15	Clga-UAA*03 allele	0	0	0	0	EU714304.1	NS
17	Clga-UAA*04 allele	0	0	0	0	EU714305.1	NS
18	Clga-UAA*05 allele	0	0	0	0	EU714306.1	NS
19	Clga-UAA*06 allele	0	0	0	0	EU714307.1	NS
20	Clga-UAA*13 allele	0	0	0	0	EU714314.1	NS
21	Clga-UAA*15 allele	0	0	0	0	EU714316.1	NS

Note: NS indicates no significance in total score, query cover, E value, and identity.

Possible implications to breeding programs for disease resistance in C. gariepinus. This study and that of Azis et al (2015b) have two things in common, namely an effort to find molecular markers that can identify and discriminate individuals of African catfish that are resistant against infection of *A. hydrophyla* (1), and they worked with the same species, namely *C. gariepinus*. They were, however, different in at least two aspects: the number of primers and the African catfish strain used. While Azis et al (2015b) decided to choose seven MHC I alleles and designed seven primer sets according to these sequences, the present study explored a threefold higher number of MHC I alleles and designed 21 primer sets correspondingly to be tested for screening. Azis et al (2015b) worked with Sangkuriang, a strain produced from a backcross of different generations of the same population (MMAF 2004; Sunarma et al 2005), while

the present study worked with Mutiara strain, a selectively bred population established from a composite base population which has undergone three generations of mass selection for growth improvement (Imron 2015). These differences seemed to have produced different results, but that may be complementary.

The previous study discovered three bands of 300, 500 and 1000 bp in sizes as promising molecular markers for discriminating resistant from susceptible Sangkuriang strains against *A. hydrophyla* infection (Azis et al 2015b). It also identified that the markers have a high similarity to MHC I allele 9 and 17 as deposited by Wachirachaikarn et al (2009). The results of the present study, in essence, have similarity to that of Azis et al (2015b), in that a promising marker was discovered. However, it was different from the previous results (Azis et al 2015a), as it was more specific as it represented a single band instead of multiple bands. The other difference was shown in the type of alleles, in which the type from the present study showed a high similarity to MHC I allele 7, instead of either allele 9 or allele 17, as reported by Azis et al (2015b). Considering the differences in the primer set and fish strain being used, the differences shown between the present and the previous studies may not necessarily be contradictory, but rather complementary.

In a different study, Azis et al (2015a) evaluated the inheritance of the MHC I allele in the first generation of the Sangkuriang strain that were challenged with *A. hydrophyla*. They found that the F1 generation produced from brooders carrying MHC resistant alleles showed higher resistance than the control. They suggested it is possible to develop Sangkuriang strain resistant to *A. hydrophyla* through a selective breeding program. The results of the present study, along with the previous ones (Azis et al 2015b; Suprapto et al 2017), have added alternative molecular markers for disease resistance, particularly against *A. hydrophyla*. For immediate use, these markers could be utilized independently in breeding programs for disease resistance to *A. hydrophyla*, at least when a breeding program works with the studied strain. However, if a breeding program will exploit the genetic of more diverse strains of *Clarias* sp., then the issue of cross-strain use of the markers needs to be resolved in advance. Molecular markers for disease resistance obtained using the Sangkuriang strain need a form of verification before use with Mutiara or other *C. gariepinus* strains. This is because different populations may experience different epidemiological conditions and evolutionary forces leading to different MHC I polymorphism (Moutou et al 2011). This is an important issue, because there was an example when a specific marker derived from one population turned out to be not applicable when it was applied to other populations, even though they belonged to the same species. This was exemplified by a sex specific marker in cyprinids. A male specific marker that was designed from and verified to be working with a cyprinid population of the Yellow river, did not work when it was used to discriminate between males and females of three other cyprinid populations (Chen et al 2009). Through a cross-strain verification, there could be widely-used molecular markers applicable to broader populations of the same species.

Conclusions. A single band and single size of MHC I molecular marker that can discriminate individuals of the Mutiara strain of *C. gariepinus* that were resistant from that were susceptible to Motile Aeromoad Septicemia syndrome caused by *A. hydrophyla* has been discovered. It was approximately 1000 bp in size, putatively part of the MHC I allele 7 and its presence was unique in surviving individuals. Patterns of inheritance of this marker in hybrids or in pure line populations need to be explored before it can be used in breeding programs for disease resistance.

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