[1] Prevention and Treatment of Diseases Caused By Fish Pathogens

2.3.10. Quantitative LAMP Method

A quantitative real-time LAMP method has been reported (Mori, et al., 2001). This method produces large amounts of the target DNA as well as an insoluble by-product, magnesium pyrophosphate, during the reaction making it possible to perform a real-time measurement of turbidity using an inexpensive photometer. Sudhakaran et al. (2008) reported the real-time LAMP assay to detect IHHNV in shrimp. The real-time LAMP method for IHHNV is simple and rapid with specific amplification within 60 min at 63ºC. The sensitivity analysis revealed this method is capable of detecting as few as 10^2–10^3 copies/μL. This method was also reported in the detection of WSSV (Mekata et al, 2009a) and YHV (Mekata et al, 2009b).

2.3.11. Conclusion

This review describes the application of LAMP method for detection of fish and shellfish pathogens. Various studies cited in this review have convincingly demonstrated that LAMP is a superior diagnostic tool compared to other methods. This method can be widely applied in clinical diagnostics, environmental monitoring and food safety in aquatic sciences.

Glossary

LAMP: Loop Mediated Isothermal Amplification,

dNTP: Mixture of dATP (deoxyadenosine triphospate) + dCTP (deoxycytidine triphospate) + dGTP (deoxyganosine triphospate) + dTTP (deoxytymidine triphospate)

3. SELECTION AND ESTABLISHMENT OF DISEASE-RESISTANT FISH

3.1. Development of Disease-Resistant Fish Using Marker-Assisted Selection

Takashi Sakamoto, Akiyuki Ozaki and Nobuaki Okamoto

3.1.1. Synopsis

In aquaculture, one way to prevent fish diseases is to develop disease-resistant strains of fish through the use of marker-assisted selection (MAS). MAS requires an understanding of the linkage between quantitative trait loci (QTL) of a target trait and DNA markers. Presently, detection of disease-resistant phenotypes requires artificial challenge tests, which are labor intensive and expensive. However, such tests are no longer needed once the linkage between disease resistance traits and DNA markers is known. So far, MAS has been used to develop Japanese flounder resistant to lymphocystis disease (LD) and Atlantic salmon resistant to infectious pancreatic necrosis (IPN).

3.1.2. Introduction

The majority of species and strains reared globally for aquaculture are relatively unimproved for commercially important traits. Presently, cultured and wild fish species

have high genetic diversity and thus have more opportunities and higher potential for genetic improvement than domestic livestock and crops which have already undergone selection over many centuries. DNA markers can be used for genetic improvement through selection of economically important traits, such as disease resistance. DNA markers detect DNA polymorphisms that can be used to trace the Mendelian inheritance of homologous chromosome segments. Economically important traits are generally thought to be controlled by many genes of small additive effects, which are known as quantitative trait loci (QTL). Construction of a genetic linkage map based on DNA markers at a large number of sites in the fish genome is necessary to identify QTLs controlling disease resistance. Once the markers associated with a QTL have been identified, it may be possible to improve other strains through introgression of the QTL through cross breeding.

One of the goals of modern selective breeding programs for aquaculture is to include the use of genetic markers from pedigreed brood stocks. This approach, called marker-assisted selection (MAS), is expected to improve the efficiency and accuracy of selection.

3.1.3. Marker-Assisted Selection

3.1.3.1. LD-Resistant Japanese Flounder (*Paralichthys Olivaceus*) In Japan

Japanese flounder is an economically important food fish that is widely cultured in Japan, Korea and China. Lymphocystis disease (LD), caused by LD virus (family Iridoviridae), has seriously damaged fish farms in these countries. There is no effective treatment for LD or a commercially available vaccine. To solve this problem, we have initiated a search for DNA markers associated with LD resistance. As a first step, our research group constructed a primary genetic linkage map in Japanese flounder using microsatellite (MS) markers. A first-generation linkage map was constructed using approx. 150 MS markers (Coimbra et al., 2003) and a more recent map has over 1000 markers (Sanchez et al., 2010).

Linkage of the DNA markers with LD resistance was analyzed in a backcross progeny (n =136) produced by crossing a susceptible male with a (susceptible x resistant) hybrid female. Fuji et al., (2006) detected a major locus (*Poli.9-8TUF*) for LD resistance on linkage group 15 on the map of Coimbra et al., (2003) (Figure 3.1.1). To introduce the trait and marker information linked to LD resistance into a commercial strain, we performed a cross between a resistant strain and a commercial strain, and generated F_1 hybrid families. The LD-resistant Japanese flounder stock produced by MAS was tested in two commercial fish farms. An allele (147bp) of *Poli9-8TUF* shows a dominant effect in Mendel's law. A new disease-resistant strain of Japanese flounder was produced by MAS using this allele. A female with LD-R that was homozygous for the favorable allele and a male from a commercial stock bred for higher growth rate and good body shape were selected as parents. A female was selected as the LD-R-bearing parent because the recombination rate of females is lower in the region where the LD-R locus is located. As expected, the favorable allele was transmitted as a heterozygote to the progeny (LD-R+ strain). The LD-R+ strain and a control strain (LD-R−) were tested at two commercial fish farms that had had LD outbreaks. The incidence of LD in the LD-R+ strain was zero

at both farms, while the incidences of LD in the control strain were 4.5% and 6.3% at the two farms (Fuji et al. 2007). LD-resistant flounder developed by MAS now account for about 35% of the retail sales of farm-raised Japanese flounder in Japan (Figure 3.1.2). Field tests of F_1 hybrid families demonstrated that LD resistance was successfully transmitted to the commercial strain.

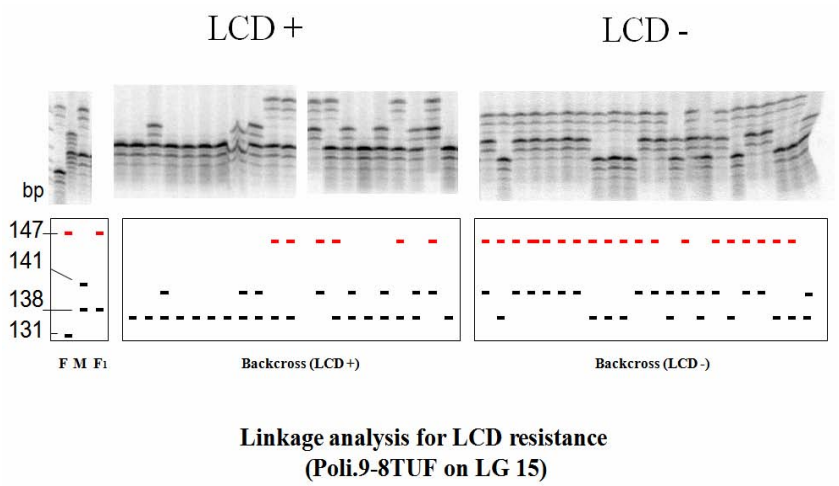

Figure 3.1.1. Autoradiograph of one marker (Poli.9-8TUF) associated with LD resistance on LG15. The upper red band (147 bp) from a resistant strain was confirmed to be responsible for LD resistance.

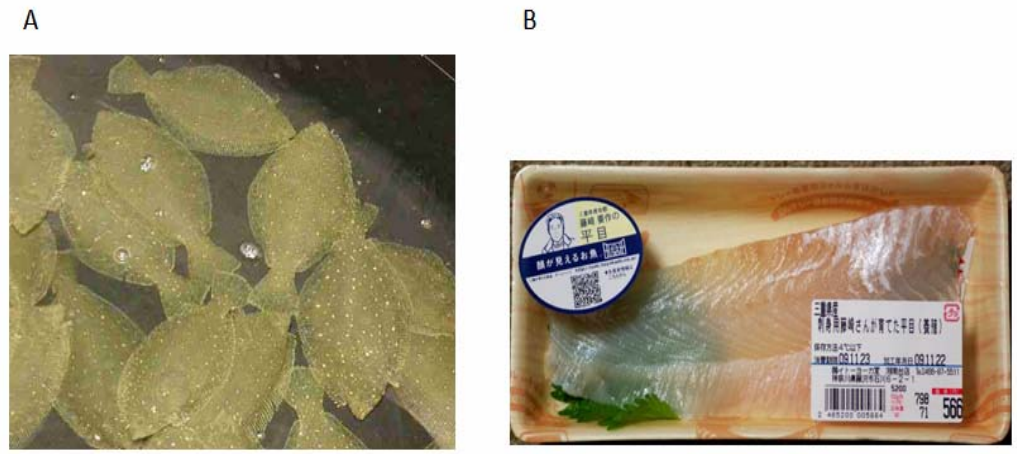

Figure 3.1.2. (A) LD-resistant Japanese flounder (*Paralichthys olivaceus*) by MAS in Japan. (B) LD-resistant Japanese flounder filet is sold at the supermarket.

3.1.3.2. IPN Resistant Atlantic salmon (*Salmo Salar*) In Norway

IPN is a viral disease that is a major problem in the production of Atlantic salmon, and other salmonid species, worldwide. In the freshwater phase of the salmon life cycle, IPN outbreaks in fry have been observed for several decades, with up to 70% mortality. In the marine environment, problematic IPN outbreaks (resulting in up to 40% mortality) have emerged more recently, coinciding with the dramatic expansion of salmon aquaculture (Houston et al., 2008). Several genetic linkage maps have been constructed for Atlantic salmon (Moen et al., 2004; Gilbey et al., 2004; Moen et al., 2008; Lien et al., 2011). One

major QTL for IPN-resistance was detected on linkage group 21 in Scottish and Norwegian Atlantic salmon populations (Houston et al., 2008; Moen et al., 2009; Houston et al., 2012). Challenge-tests showed that the QTL had the same beneficial effect on fry as on post-smolts, with the confidence intervals for the QTL positions in the two age groups overlapping. QTL genotypes based on MS markers and single nucleotide polymorphism (SNP) markers were deduced within most parents of breeding companies, providing a solid framework for linkage-based MAS within the whole population in subsequent generations (Moen et al., 2009; Houston et al., 2012).

3.1.4. Future Perspectives

The culture of some Japanese species such as Pacific bluefin tuna, yellowtail and Japanese eel is still based on the capture of wild fish. Recently, methods for propagating these species in captivity have been developed. Now there is a need to develop useful strains from the wild fish populations. We have attempted to combine classical selection and marker-assisted selection (MAS) in yellowtail (*Seriola quinqueradiata*) to develop strains resistant to the ectoparasite *Benedenia seriolae*, which causes secondary infections and reduced growth.

First, we constructed a genetic linkage map for this species. Second, we obtained the first generation by classical selection, examining 200 adult wild yellowtail individuals from coastal waters for *B. seriolae* and selecting a few fish with low numbers of parasites. These fish were one-on-one crossed to produce F_1 families. In the F_1 families, we searched for D NA markers associated with resistance to the parasitic infection and identified two QTLs (Squ2 and Squ20) (Ozaki et al., in press). Third, MAS was performed using QTL markers associated with parasitic resistance to produce F_2 families. F_1 siblings were placed in two groups according to whether or not they inherited the QTL alleles for resistance. Then, two types (putative resistant families and susceptive families) of F_2 families were established by one-on-one crossing. The two types of families were reared together and challenged by exposure to *B. seriolae*. In the F_2 families, the resistant family fish had significantly fewer parasites than the susceptive family fish in all tested cases. These results show that it is possible to establish a new strain with resistance to some diseases by combining classic breeding and molecular genetic breeding (MAS).

Glossary

IPN: nfectious pancreatic necrosis,

LD: Lymphocystis disease,

MAS: Marker-assisted selection, MS: microsatellite,

QTL: Quantitative trait loci, SNP: single nucleotide polymorphism

3.2. Establishment of Disease-Resistant Fish

Ryosuke Yazawa

3.2.1. Synopsis

The establishment of disease-resistant strains for aquaculture is important since

infectious diseases are the greatest problem in the aquaculture industry all over the world. There are several ways to prevent and control diseases. Drugs and vaccines are the most popular and direct methods to control infectious diseases; however targeted species and diseases have been limited, besides they are both expensive and labor-intensive. Although, selective breeding is the traditional way to establish new strains, the rate and consistency of genetic improvement might be unstable. Transgenic technology could be an alternative approach to prevent and control infectious diseases, since it is theoretically possible to integrate the foreign gene coding the protein or peptide that could provide a desirable trait to the host species. Most research to have successfully established disease-resistant transgenic strains is based on the idea of overproducing the antimicrobial peptide that possesses anti-bacterial or anti-viral activities derived from the transgene. In this section, recent advances in the fish transgenesis for disease-resistance are discussed.

3.2.2. Introduction

Infectious diseases are the one of the greatest problems for aquaculture and causes severe economic losses worldwide. Therefore, it is necessary to establish the disease-resistant strains for aquaculture species. To address this issue, transgenic technology could be an approach for prevention and control of infectious diseases as an alternative to the conventional methods, such as a selective breeding. Transgenesis is the process of introducing an exogenous gene, called a transgene, into a host species so that the host species acquire a new trait derived from the exogenous gene and transmit the trait to its offspring. Fish transgenesis could be a way to establish new strains more rapidly and consistently rather than the traditional selective breeding. Besides, selective breeding may also have the potential risk to retain undesirable traits. Transgenic fish with enhanced disease resistance would increase the production efficiency and benefit the aquaculture industry. To achieve this task, several researchers have been tried to establish disease-resistant fish strains (Dunham, 2009).

3.2.3. Transgenesis for Disease-Resistance

To date, most research to have successfully established disease-resistant transgenic strains is based on the idea of over-expressing an antimicrobial (anti-bacterial or anti-viral) peptide gene (summarized in Table 3.2.1). Although the inhibition of viral replication by antisense RNA is also a potential technique to prevent viral diseases, thus far there is only one report of the transient expression of an antisense of viral genes improving viral resistance in rainbow trout (Anderson et al, 1996).

Anti-bacterial or –viral peptides play important roles in the innate immunity of a wide range of organisms. Although fish possess their own antimicrobial peptides against infections from pathogenic organisms, antimicrobial peptides from different taxa tend to possess higher activities in a xenogeneic environment. Therefore, the genes coding these antimicrobial peptides were chosen as a transgene to produce disease-resistant transgenic fish. It seems reasonable that the host species do not have effective antimicrobial peptides against the pathogens possessing high virulence to the host species from the viewpoint of the evolutionary aspects to the host-pathogen relationship. Besides, antimicrobial peptides that possess activity against a wide spectrum of bacteria, such as cecropin,

lysozyme, hepcidin or lactoferrin, have been chosen as the transgenes in the previous studies (Table 3.2.1).

Species	Foreign gene	Promoter	Challenge test	Ref.
Channel catfish	Silk moth cecropin	CMV	*Flavobacterium columnare*	Dunham et al, 2002
Medaka	Silk moth cecropin Pig cecropin	CMV	*Pseudomonas fluorescens, Vibrio anguillarum*	Sarmasiket al, 2002
Grass carp	Human lactferrin	Carp beta-actin gene	*Carp haemorrhage virus*	Mao et al, 2004
Zebrafish	Chicken lysozyme	Japanese flounder keratin gene	*Flavobacterium columnare, Edwardsiella tarda*	Yazawa et al, 2006
Rare minnow	Rare minnow MX	CMV	*Grass carp reovirus*	Su et al, 2009
Zebrafish Convict cichlid	Tilapia hepcidin	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Hsieh et al, 2010
Zebrafish	Epinecidin-1	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Peng et al, 2010
Atlantic salmon	Rainbow trout lysozyme	Ocean pout antifreeze protein gene	-	Fletcher et al, 2011
Zebrafish	Tilapia hepcidin Giant tiger prawn chelonianin	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Pan et al, 2011

* CMV: cytomegalovirus promoter

Table 3.2.1. List of the disease resistance transgenic fish reported to date.

For the effective action of the transgenes in the host species, it is essential to control their expression at a high level or tissue-specific manner using the regulatory region of the genes, called the promoter. In the beginning of the transgenic fish studies, the promoter derived from viruses, such as the CMV promoter, were frequently used due to their high activity in the broad range of species. It is preferable to use promoters derived from target fish (or closer species) as recent studies suggest, since the promoters derived from fish are thought to work more dependably in general.

These transgenic strains when challenged with bacteria showed resistance against the pathogens. In the case of our previous work, we established a transgenic zebrafish that expressed the chicken lysozyme gene under the control of the Japanese flounder keratin gene promoter (Su et al, 2009). In challenge experiments, 65% of the F2 transgenic fish survived an infection of *Flavobacterium columnare* and 60% survived an infection of *Edwardsiella tarda*, whereas 100% of the control fish were killed by both pathogens. Thus, new strains with enhanced disease resistance by genetic modification have been successfully established.

However, there is no study to establish the disease-resistant transgenic fish in marine

aquaculture species. The generation of transgenic fish targeted on marine aquaculture species is still not popular due to the difficulties associated with handling small and fragile pelagic eggs. Recently, our group has developed a feasible and reproducible microinjection method for the pelagic eggs of marine fish and to establish stable transgenic strains in Nibe croaker, *Nibea mitsukurii* that could be a model species for the marine aquaculture fish species spawning pelagic eggs (Yamamoto et al, 2011). Accumulation of these techniques will realize the production of disease-resistant transgenic aquaculture species in near future.

3.2.4. Risks and Benefits of Transgenic Fish

Although, fish transgenesis has great advantages for the breeding of aquaculture species, there are several potential risks, particularly environmental and human health concerns. If transgenic fish escape into the natural environment, it would cause problems ecologically and genetically. Sterilization of transgenic strains with the polyploidy treatment and/or physical containment by the land-based marine aquaculture with the closed re-circulating system could be realistic way to solve this problem (Dunham, 2009). Another issue is human health concerns. To settle this issue, it is important to select the targeted gene, to conduct food safety trials securely and to keep consumers informed. Since it might be possible that transgenic fish with enhanced disease resistance may decrease or suppress the drug usage in aquaculture, this would improve the aquaculture production more safely from the standpoints of the drug residues and the emergence of antibiotic-resistant pathogens. Although it is essential to guarantee the safety of transgenic fish as genetically modified food, the disease-resistant transgenic fish could be of great help to improve the aquaculture.

Glossary

CMV: Cytomegalovirus

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