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Genetically Modified, Red Fluorescent Zebrafish: Detection, Crossing, Inheritance of Red Fluorescence, and Tolerance to Low Temperatures

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Under Cartagena Protocol domestic law, genetically modified (GM) fish have not yet received permission to be imported or sold in Japan. However, it is anticipated that ornamental, fluorescent GM fish will be imported and sold prior to any official process for evaluating their effects on biological diversity as GM organisms (GMO). Therefore, we have devised a method for detecting GM fish that utilizes PCR for the detection of replication origins of plasmids that generally exist as transgenes integrated in the GMOs' chromosomes. Using red fluorescent zebrafish (rfZF), sold in Japan, and *rpsL* GM ZF established by us, we demonstrated the feasibility of this method for detecting GMO. Next, we examined the biological characteristics of the GM rfZF. They were able to cross with non-GM ZF; the red fluorescence was inherited by their progeny according to expected outcomes, based on Mendelian genetics. Examination of the low temperature tolerance of rfZF and non-GM fish indicated that the lethal, minimum temperature was the same for both fish (5°C). These findings suggest that the transgene of the rfZF could spread among wild-type ZF, and that these ZF could overwinter in the southernmost part of Japan. We conclude that a continuous monitoring is required.

Key words: genetically modified fish, red fluorescent zebrafish, temperature tolerance

1. Introduction

A number of genetically modified zebrafish (*Brachydanio rerio*) (GM ZF) and medaka (*Oryzias latipes*) have been developed by introducing fluorescent protein genes linked to different tissue specific promoters into fish. This transgenic technology has also been applied to develop novel, ornamental GM fish with intense fluorescence. Chou et al. produced GFP medaka with intense fluorescence[2], and Gong et al. introduced GFP, RFP, and YFP into ZF[4]. The latter GM fish named GloFish was commercialized in the USA (http://www.glofish.com). The GFP medaka, named TK-1, and the GFP/RFP ZF, named TK-2, are sold in Taiwan (http://www.azoo.com.tw/). ZF and medaka are indigenous to South Asia and East Asia, respectively, and are distributed broadly in these areas. Once these GM ZF or medaka are released outside of laboratories, they could potentially influence the biological diversity of native fish by crossing with native species and competing with them for survival, resulting in an alteration of the ecosystem.

In Japan, under Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Protocol domestic law), GM fish have not received permission for importation or sale since the law was enacted in 2004 (http://www.bch.biodic.go.jp/english/law.html). However, it is anticipated that GM fish, most notably ornamental, fluorescent fish, will be imported and sold without any official process for evaluating their effects on biological diversity as GM organisms (GMO)[5]. Actually, red fluorescent-colored ZF (rfZF) of probably GMO origin were imported and sold in a pet shop in early 2007. These rfZF were obtained by the Ministry of the Environment of Japan and were shown to be GMO, as they contained the DsRed 2 (Clontech Laboratories, Inc., CA, USA) transgene as judged by researchers in the Fisheries Research Agency of Japan. In light of this, a procedure for detecting GM fish without any prior information regarding the integrated transgene is required. In the present study, we established a procedure for judging whether or not fish suspected of being GMO are genetically modified without possessing any prior information regarding their specific transgenes. Moreover, using rfZF, we also examined the inheritance of red fluorescence in progeny fish, the ability of rfZF to crossbreed with native ZF, and the low temperature tolerance of the rfZF. All of these properties are likely to influence the effect of any released rfZF on the biological diversity of native species in Japan.

2. Materials and Methods

2.1. Zebrafish

rfZF suspected to be GMO were obtained from a pet shop

by the Ministry of the Environment of Japan. They were originally examined in the Institute of Fisheries Research Agency of Japan where researchers demonstrated the presence of the DsRed 2 transgene in the rfZF. *rpsL* GM ZF, which had been established by us using AB line of ZF and maintained for over 10 generations, carries approximately 350 copies of the pML4 plasmid in their chromosomal DNA[1]. As non-GM fish, ZF AB line was used. All kinds of fish were maintained in rearing water (0.1% Instant Ocean salts) at 24–25°C with a 14 h light/10 h dark cycle.

2.2. Polymerase chain reaction (PCR)

Primer sets used for amplification of ori and ZF beta-globin chain were [forward 5'-ACGAGGGAGCTTCCAGGGGGA-3' and reverse 5'-TCGTTCGGCTGCGGCGAGCGGT-3'] and [forward 5'-AGGTGTCTCATCGTGT ACCCCT-3' and reverse 5'-CTTACCCTGAAGTTGTCGGGA-3'], respectively. The primer set for detecting both DsRed1 and DsRed2 was [forward 5'-TGTCCCCCAGTTCCAGTACG-3' and reverse 5'-CGATGGTGTAGTCCTCGTTGTG-3'].

We performed PCR reactions in a final volume of 20 microL of rTaq polymerase reaction mixture (attached rTaq polymerase) containing additionally 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.1 microgram DNA, 20 pmole of each primer and 1 unit of rTaq polymerase (Toyobo Co. Ltd., Osaka, Japan). The reaction conditions were 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. PCR products were separated by agarose gel electophoresis and visualized with ethidium bromide. As a positive control, PCR against the beta-globin chain was performed simultaneously to confirm that the PCR worked properly.

2.3. Temperature Tolerance Experiment

Four month old F_1 fish and AB line were used. F_1 fish with red fluorescence (distinct red and light red (orange)), and without fluorescence (yellow fish) had been obtained by mating males and females rfZF. Seven fish of each type were put into 2-L of rearing water in plastic tanks. These tanks were placed in temperature-controlled incubators (Sanyo Electric Co., Ltd., Osaka, Japan), and the temperature was decreased 1°C per day. Fish were fed Tetramin tropical flake food once a day. Aeration was performed once a day, and half of the rearing water was changed twice a week.

Ten hatched fry (post-fertilization day, pfd, 6), which were hemizygous rfZF obtained by mating rfZF ID No. 3 and fish from the AB line, were put into 0.3-L of rearing water in a plastic tank and placed in the same incubator as described above, in which the temperature was decreased 1°C per day. Fry were fed once a day a kind of zooplankton, termed Rotifera, prepared in our institute. Aeration was performed once a day, and the rearing water was exchanged once or twice a week. After 2 weeks, when the temperature of the incubator had decreased to 10°C, 5 of the 10 fry were returned to the tank at 24°C, and the other 5 fry were kept in the low temperature tank. The former 5 fry were fed both Rotifera and brine shrimp (*Artemia franciscana*). The phenotypes of all these fry were red and black striped.

We checked all the fish in the incubator at least once a day. With the decrease in temperature, fish gradually reduced their movements and finally came to rest at the bottom of the incubator. The fish were considered to be dead when their hearts stopped beating and/or when they were unable to recover after return to a higher temperature.

3. Results and Discussion

3.1. Visual observation of rfZF

Ten rfZF brought into the National Institute for Environmental Studies had no black stripes and differed slightly in color; some looked distinctly red, while others were light red or orange (Fig. 1). A large number of progeny, most of which were red-colored, were obtained by mating, indicating that the rfZF fish could reproduce. A few yellow fish without red fluorescence were found in the progeny, indicating that rfZF had been produced from mutant yellow (sometimes called golden) ZF, and that there were hemizygous and homozygous fish in the rfZF population.

3.2. Detection of GM fish by PCR for the replication origin of the plasmid

A transgene is generally prepared as a plasmid, a 'transgene construct', which carries the actual transgene sequence (protein coding sequence linked to a promoter), a selectable marker such as an antibiotic resistant gene and an origin of replication (ori). To prepare a large amount of the 'trans-

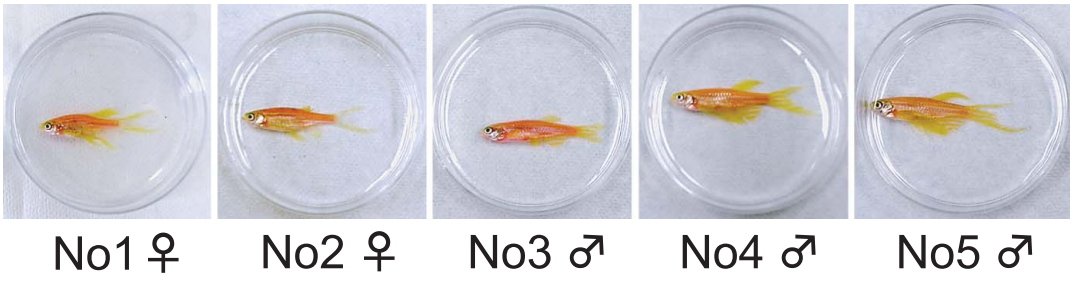

Fig. 1. Red fluorescent ZF used in this study.
Numbers 2 and 3 appeared distinctly red, and numbers 1, 4 and 5 looked light red or orange. The sex of each fish was established after mating with non-GM fish.

gene construct' for establishing the GMOs, plasmids with efficient ori are generally used. To our knowledge, almost all of the plasmids currently used for large scale preparations contain the ori derived from colicin E1 (ColE1); pBR and pUC plasmids bear either the ColE1 ori or the ColE1 ori with a single base substitution, respectively. GM fish are generally established by microinjection of the 'transgene constructs' into fertilized eggs, followed by selection of germ-line transformants. Therefore, most established GM fish lines possess plasmid-derived ori sequences integrated into their chromosomal DNA.

We designed PCR primers for detecting both types of ColE1 ori. As shown in Fig. 2, amplified products were observed in DNA prepared from two kinds of GM fish, rfZF and *rpsL* GM ZF, and from *E. coli* bearing the plasmid pUC19, whereas no products were amplified using the DNA of the negative control, i.e. non-GM fish. We confirmed that the amplified product in the rfZF DNA sample was obtained from the ColE1 ori by DNA sequencing.

To confirm that PCR had proceeded correctly, PCR against the beta-globin chain was performed at the same time. An amplified band of the beta-globin chain was observed in the negative control samples (non-GM fish), suggesting that PCR had not been inhibited in these samples. Thus, GM fish containing the ColE1 ori can be distinguished from non-GM fish by PCR against the ColE1 ori. Theoretically, this ori-directed PCR is also applicable for detecting other GMOs carrying a plasmid vector, but not for GMOs with a phage vector or a retrovirus vector.

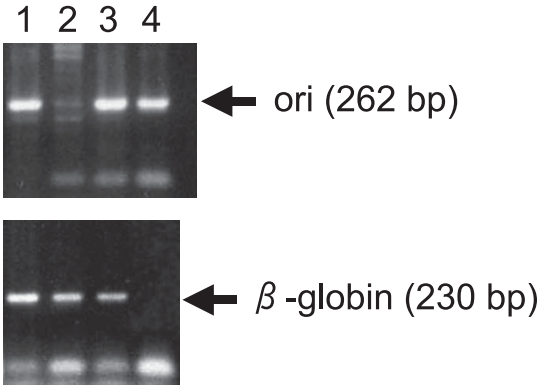

Fig. 2. PCR amplification of the plasmid replication of origin and beta-globin gene.
Genomic DNA prepared from the following samples were subjected to PCR.
Lane 1, rfZF; lane 2, non-GM fish (AB line); lane 3, *rpsL* GM fish; lane 4, DNA from *E. coli* bearing pUC19.

3.3. Crossing rfZF with native ZF and the inheritance of red fluorescence

To examine the crossbreeding of rfZF with native ZF and the inheritance of red fluorescence, each of 5 rfZF was crossed with ZF from the AB line. We succeeded in obtaining offspring from all the breeding combinations. All the offspring possessed black stripes, which is consistent with the observation that pigment mutants are generally recessive. Approximately half of the offspring of fish ID1, 4 and 5 were red and almost all of the offspring of fish ID2 and 3 were red (Table 1), indicating that their parents were hemizygous and homozygous with regard to the red fluorescence phenotype, respectively. When a hemizygous male and a hemizygous female were crossed, approximately 75% of the fish had a red body color (Table 2). Thus, the red fluorescence phenotype was inherited, as expected, according to Mendelian genetics.

Several offspring obtained from each combination were examined to determine whether they carried the DsRed transgene by PCR. As shown in Fig. 3, the red fish body color was consistent with the presence of the DsRed transgene in these fish. All red-colored fish contained the DsRed sequence, whereas the non-colored fish did not. These findings indicate that the DsRed gene was integrated stably into the chromosome of vfZF.

3.4. Temperature Tolerance Experiment

Four types of fish (7 fish for each type) were utilized in experiments to compare the low temperature tolerances of

Table 1. Inheritance of red fluorescence after crossbreeding red fluorescent ZF with the AB line.

F_0		F_1[a]					
ID	Sex	No. of F_1 fish obtained	Phenotype				Genotype F_0 parent[b]
			Black stripes		Red fluorescence		
			No. of fish	%	No. of fish	%	
1	F	39	39	100	22	56	hemi
2	F	44	44	100	44	100	homo
3	M	111	111	100	110[c]	99	homo
4	M	108	108	100	48	44	hemi
5	M	158	158	100	72	46	hemi

[a] F_1 were obtained by crossing each F_0 with several non-GM ZF of the AB line.
[b] The genotype of the F_0 parent was determined from the percentage of rfZF in the F_1 generation.
[c] One fish without red color was seen. We concluded that it was probably a contaminant from the non-GM F_1 generation of other crossings, because in the same morning we also collected non-GM fertilized eggs from crossings between non-GM fish.

Table 2. Inheritance of red fluorescence after crossbreeding hemizygous red fluorescent ZF.

rfZF parents		Offspring				
			Phenotype			
Female	Male	No. of offspring obtained	Black stripes		Red fluorescence	
			No. of fish	%	No. of fish	%
F_0 No. 1	F_0 No. 4	10	0	0	7	70
F_1 Orange	F_0 No. 5	64	0	0	52	81

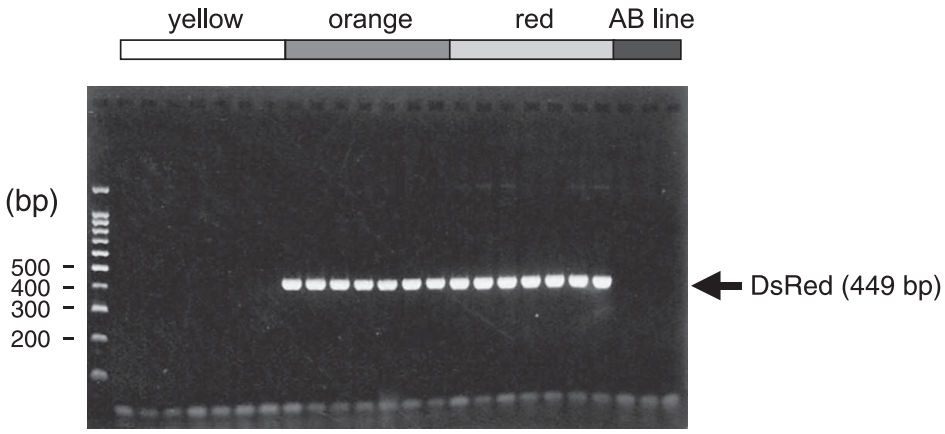

Fig. 3. PCR amplification of the DsRed gene.
Genomic DNA samples from F_1 fish, whose body colors were yellow, orange (light red), and distinctly red, and non-GM fish (AB line) were subjected to PCR.

the GM and non-GM fish. Three types consisted of the F_1 fish of rfZF: these were distinct red fish (rfZF), light red (orange) fish (rfZF) and yellow fish (non-GM), obtained by mating rfZF. The remaining type was the AB line. The temperature was decreased 1°C per day, mimicking the temperature change in autumn and winter in Japan. When the temperature reached 10°C, all 4 types of fish stopped swimming actively and started to eat poorly. At 7°C, all types of the fish did not move at the bottom of the tank unless startled. At 5.5°C, 5 fish out of 7 of the AB line were dead, while the other three types of fish were alive. The differences in sensitivity to low temperature may be explained by the fact that the AB line is an inbred strain and the others are probably not. At 4.5°C, all the fish were dead (Fig. 4). No differences in low temperature tolerance were observed between distinct red fish (rfZF), light rfZF and yellow non-GM fish. These findings are consistent with a report by Cortemeglia and Beitinger[3], which found relatively small absolute differences in mean temperature tolerances between wild-type and transgenic ZF (Glofish) (5.3°C and 5.6°C, respectively), following an initial acclimation to 20°C.

The F_1 hemizygous hatched fry (post-fertilization day, pfd, 6) obtained by mating rfZF ID No. 3 with non-GM ZF (AB line) were challenged with cold by decreasing the temperature of the incubator by 1°C per day. The temperature control fry, obtained at the same time, were kept at 25°C. Although both fry were fed similarly, their sizes were significantly different after 2 weeks; the median length of the control fry was approximately 10 mm or above, while the median length of the fry in the cold incubator was approximately 7 mm. At 11°C, the fry started to swim unusually, displaying oscillatory movements. On the following day, at 10°C, 5 fry out of 10 were returned to the tank at 25°C. The remaining 5 fry were exposed to further decrements in temperature. At 8°C, one of the 5 fry died, and the remaining 4 fry died by the time the temperature was reduced to 6.5°C (Fig. 5). When compared to adult fish, both the rfZF and AB line fry were more sensitive to cold temperature (Fig. 5).

To examine the reproductive ability of the cold-exposed fry, the fry that had been exposed to cold temperature and then returned to the normal rearing temperature (25°C) were reared to adulthood. Several cold-exposed females were mated with native males (AB line), and several cold-exposed males were mated with native females. Both mating groups successfully produced fertilized eggs, and the eggs developed normally, suggesting that exposure to cold, produced by gradually lowering the temperature to 10°C, is not necessarily detrimental to the reproductive capacity or development of ZF.

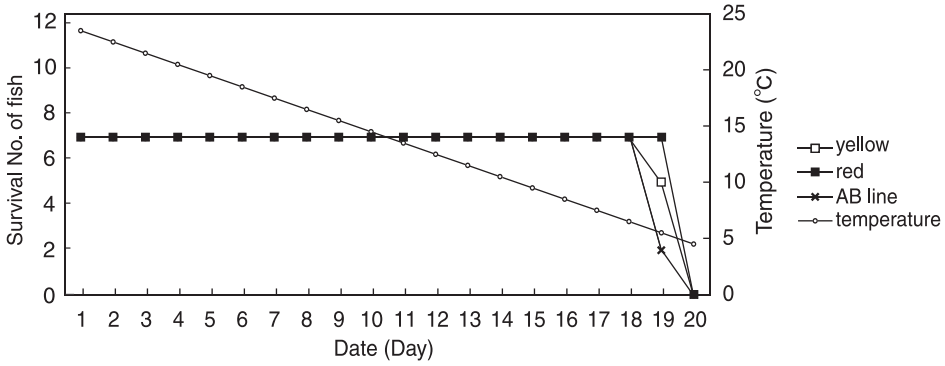

Fig. 4. Low temperature tolerance of red fluorescent ZF.
Survival curves of three groups of fish with different body colors (7 fish for each group, including distinctly red, yellow and black striped fish) are shown. The curve of the light red (orange) fish is not shown since it was the same as that of the distinctly red fish. The temperature of the incubator was decreased 1°C per day.

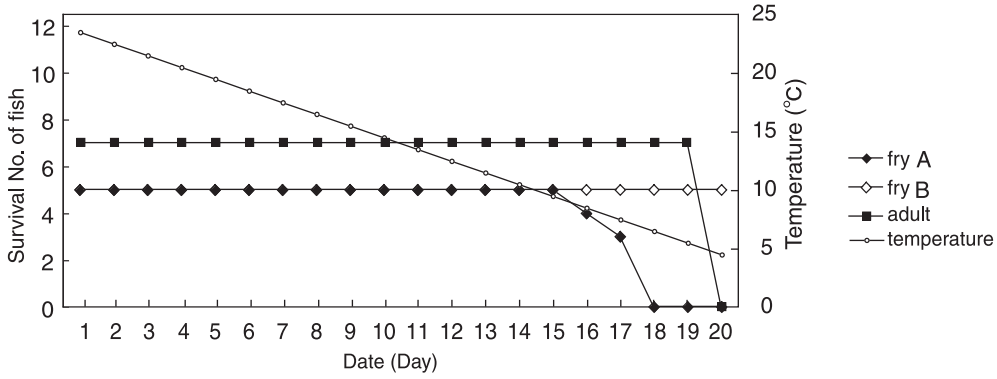

Fig. 5. Low temperature tolerance of F_1 hemizygous fry.
F_1 hemizygous fry, identified by the red fluorescent color and black stripes, were obtained by mating rfZF and non-GM fish (AB line). Ten F_1 fish were divided into 2 groups of 5 fish each; one was challenged with continual drops in temperature until death (fry A), while the other group was returned to normal temperature after the temperature in its incubator reached 10°C (fry B). The survival curve of the adult F_1 rfZF from Fig. 3 is shown for reference.

4. Conclusion

4.1. Detection of GM fish without information regarding the GMO

Based on the results obtained in this study, we propose that the described method is useful for judging whether or not fish suspected of being GMO are, in fact, genetically modified, because almost all ornament GM fish may have been established utilizing plasmid vector.

DNA, generally most-readily extracted from the fin, is utilized as a template in PCR, using primers for the ori and beta-globin chain. The amplification of ori confirms that the fish is a GMO. Furthermore, identification of the integrated transgene is desirable to confirm this conclusion. When no amplification of ori is observed, but amplification of globin is observed, the fish is likely not a GMO. When amplification of neither the globin gene nor the ori is detected, there is a problem either with the extracted DNA sample and/or the PCR reaction. In these cases, new DNA extracts should be prepared again, and/or the PCR mixtures should be prepared again more carefully before performing the reactions again.

4.2. Potential risk for biological diversity

We demonstrated here that rfZF are able to cross with native ZF. Furthermore, we showed they could not survive below a temperature of 4.5°C, but that they could live at 6.5°C for at least one day. Moreover, fry exposed to cold temperature (10°C) were able to grow to adulthood and normally reproduce. These findings indicate that the transgene in the rfZF could spread among wild-type ZF, and that rfZF, as well as wild-type ZF, could overwinter in the natural water environments of Japan, for example, in the southernmost part of Japan or near hot springs. Continuous surveillance is required to avoid spreading either the transgenes among natural, non-GM ZF or the GM fish themselves.

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