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Novel PCR-based assay for rapid identification of Red Fluorescent Proteins in GloFish and GloFish x wildtype zebrafish (*Danio rerio*) hybrids

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Abstract
Twenty-five zebrafish presenting three different colour variants were collected and analysed by molecular markers in order to define their species and to verify if the unusual colouration presented by two morphotypes was due to genetic engineering or were natural colour variants obtained by selective breeding or spontaneous mutations. The three morphotypes collected were (a) an unusual reddish-pink (suspected red GloFish®), 15 specimens confiscated by the operative ecological core of police officers; (b) the black/blue striped colouration typical of wildtype *Danio rerio* (five specimens) and (c) an hybrid morphotype with an atypical flashing red pigmentation of skeletal muscle and a blue-black striped pigmentation of skin (five specimens obtained by crossing suspected GM danios with wildtype line) obtained by crossing wild type danios with fish confiscated by police.
Despite the three different colourations presented, all analysed samples were unequivocally *Danio rerio*, excluding the possibility of belonging to different species or natural variants, which should carry different skin colours. Except the five wild-type danios, all other samples analysed should belong to an engineered variant and thus forbidden by EU GMO legislation. Here we designed a new highly reliable and affordable PCR-based assay able to identify the presence of red fluorescent proteins not only in the 15 parental GM samples but also in the five F1 hybrids created *ad hoc*.
The cross-breeding experiment clearly demonstrated that the exogenous construct made by red fluorescent protein under a fast-muscle promoter was inherited by F1. For this reason in our opinion also cross-hybrids obtained by GM danios and wildtype zebrafish should be included in the list of organisms regulated by GMO legislation.
Finally, the obtained results suggest that the PCR-based assay here implemented could represent a cost-effective test to rapidly identify suspected GM fish, which carry RFP/dsRED isoforms.

Keywords: *GMO, Red Fluorescent Protein, GloFish,* Danio rerio, *forensic genetics*

Introduction

The zebrafish *Danio* (= *Brachydanio*) *rerio* is a tropical freshwater fish, which belongs to the family of Cyprinidae. Different closely related species within the genera *Danio* typically exhibit a wide variety of colourations and pigment patterns including horizontal stripes, vertical bars and others (Meyer et al. 1993). This fish is considered a good species for beginner aquarists and for this reason a number of additional colours and morph-variants were developed for the aquarium industry. The latest novelty in this field is represented by the so-called GloFish®, a patented genetically modified zebrafish obtained by insertion of fluorescent protein genes (GFP, YFP or RFP/dsRed) under a strong muscle-specific promoter (e.g. like that of the *mylz2* gene, Gong et al. 2003; Zeng et al. 2005). The genetically modified (henceforth GM) danios were initially developed as a model for tissue/organ development, toxicological and gene expression pattern studies, for the analysis of regulatory elements of gene promoters/enhancers, for use as bioreactors and for several other applications (Gong et al. 2001). Successively, the transgenic technology based on the insertion of "living colour"

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fluorescent proteins were used to develop novel varieties of ornamental fish and several stable lines of transgenic zebrafish expressing green fluorescent protein (GFP), red fluorescent protein (RFP), or yellow fluorescent protein (YFP) in skeletal muscle were obtained and registered as trademark (Gong et al. 2008). These transgenic danios display fluorescent (glowing) colours that are steadily visible under normal light conditions (and emphasized under UV wavelengths), a result particularly appreciated by aquarists (Gong et al. 2003). Recently, the same technology has been applied to other freshwater aquaria species, such as medaka *Oryzias latipes* (Zeng et al. 2005). The transgenic zebrafish and medaka are generated by microinjection of plasmid containing the synthetic construct made by GFP, RFP or YFP (or combinations of them) under the *mylz2* promoter into 2–4 cell embryos (Ju et al. 1999). The GM danio lineages obtained applying this protocol are very stable and standard breeding experiments have confirmed the typical Mendelian inheritance ratios of the transgenic construct (Gong et al. 2003).

Since the severe politic of risk regulation in Europe, a wide array of European consumer and environmental regulations, including those governing the genetically modified organisms (GMOs), are more restrictive than in the United States or in the East Asian countries. The import, sale and possession of GMOs is strictly regulated in the EU countries and the release and marketing of genetically modified ornamental fishes is controlled mainly under the Council Directive 2001/18/EC (Plan & Van den Eede 2010), which states that the marketing or release of any GM ornamental fish is prohibited unless authorisation has been given by the *Environmental Protection Agency*. There are currently no genetically modified fish authorised for release or marketing in Europe or Great Britain therefore anyone who knowingly releases or markets such fish is committing an offence under The Environmental Protection Act 1990. In Italy the above-mentioned Directive is implemented by the Legislative Decree 224/2003, which establish severe penalties and administrative sanctions up to 51,700 € for people who posses or trade GloFish or other genetically modified ornamental fishes. Similar regulations have been implemented in UK, Northern Ireland, Scotland, Wales and California.

Starting from 2006, the authorities of England, Germany, Netherlands and Italy reported that brightly coloured zebra danios genetically modified to fluoresce under white and UV light had been identified for sale from several retailers.

The development of tests, which are able to detect the presence of exogenous fluorescent proteins, has become essential for authorities to take appropriate action. The easiest and cheapest diagnostic strategy is to expose suspected GM fish to a UV light source: zebra danios containing fluorescent proteins will fluoresce under ultraviolet light (400–320 nm) whereas non-GM zebrafish will not fluoresce. Nevertheless, this test is not decisive because fluorescent dyes may have been absorbed or fed to the fish and produce the "*GM-like*" glowing colouration (Auctorum 2007). Tests based on excitation spectra of suspected GloFish (expected to be 508 nm and 583 nm respectively for GFP and RFP) are not conclusive for the same reason. Molecular assays based on identification of transgenic construct via Polymerase Chain Reaction (PCR) by using transgene-specific primers overcome the problem of possible alternative causes of flashing colouration by seeking directly the fluorescent transgene in fish genotype.

Two couples of primer pairs targeting RFP by PCR are currently available in the literature (Ji et al. 2005; Rehbein & Bogerd 2007) but these two RFP primer sets are not suitable for an unambiguous identification of red fluorescent protein variants by marker amplification and sequencing (detailed explanation in the Discussion section).

In this work 25 zebrafish presenting three different colour variants were collected. The morphotypes were (i) an unusual reddish-pink (suspected GM fishes, 15 specimens) and (ii) the black/blue stripped colouration typical of wildtype danios (five specimens) and (iii) a third hybrid morphotype obtained *ad hoc* by crossing GloFish and wildtype danios (description given in Results, five specimens). Despite the existence of GloFish of different colours, this study focuses only on red morph-variant because the biological samples with an unusual colouration here analysed were either confiscated by police or were progeny of fish confiscated by police itself, and only the GM red variant was available for molecular analyses. Any result, which was not possible to test against a positive control, was excluded.

Samples were analysed by genetic molecular tools in order to (i) define the belonging species and (ii) to identify if the origin of the unusual colouration presented by two out of three morphotypes, which can be obtained by natural processes (selective breeding or spontaneous mutations) or genetic modifications.

Materials and methods

Sample collection, fish crossbreeding

A total of 25 specimens were collected. Fifteen fishes (seven males and eight females) suspected to be genetically modified because of the unusual

colouration were collected in Italy from ornamental fish wholesalers in 2010. Five wildtype D*anio rerio* specimens were collected in Italian aquarium retailers as negative control. Due to the large amount of fingerling coming from a single breeding pair, the experiment was performed crossing single couples made by four wild long finned males with four GM females. Breeders were kept separately until the coupling, then each pair was reared in four different 4L tanks at 26°C, pH 7.4 and fed on frozen adults of *Artemia* to induce spawning. The breeders were successively removed in order to prevent eggs predation. Tanks were connected to a recirculation system equipped with a mechanical filter to maintain the optimal water quality. Photoperiod was set up to simulate 13 hours of brightness and 11 hours of darkness. The obtained larvae were fed on food for live bearing fishes Liquifry 2 (Interpet) and newly hatched brine shrimp (*Artemia* nauplii). Eight days after hatch, the *Artemia* nauplii were integrated with dried pellet. Five interbred specimens obtained from the crossing of a single parent pair were ethanol preserved for the following genetic analyses.

DNA extraction, species validation

DNA was extracted starting from 25 mg of fresh tissue with DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Molecular determination of belonging species was necessary to clearly demonstrate that analysed fishes with the uncommon colouration were *Danio rerio* genetically modified and not a different species which phenotype occurs in nature as a flashing red colouration. Since morphological features were not sufficient for this purpose, the belonging species was validated by molecular means cycle sequencing partial Cytochrome B and 16S rDNA genes in five specimens for each morphotype.

System for red fluorescent protein detection

To check the amplificability of the nuclear DNA, a parvalbumin gene fragment of 220 bp was amplified using the IFF primer system according to Rehbein and Bogerd (2007). All suspected genetically modified samples were first checked for presence of Red Fluorescent Protein (RFP) using the 2206/2207 primer couple according to Rehbein and Bogerd (2007). Sequence obtained, sequences of vector pDsRed2-N, transgenic zebrafish G1 and G2 (Vektor 1 2206, ZEBGV 2206 and ZEBGV 2 2206 respectively, Rehbein & Bogerd 2007) and 25 synthetic constructs containing RFP (Table I) downloaded from NCBI databank were aligned using MEGA 4.0.

The following new internal amplification and sequencing primers specific for RFP detection on different constructs were designed on conservative sites with Primer 3 (available at http://frodo.wi.mit.edu/primer) and tested in silico by Amplify 3.0 (http://engels.genetics.wisc.edu/amplify):

2-RFP_F: 5′- GAAGCACCCCGCCGACATCC -3′ (amplification primer)
4-RFP_F: 5′- GAACTTCGAGGACGGCGGCG -3′ (amplification primer)
7-RFP_R: 5′- GGCAGCTGCACGGGCTTCTT GGC -3′ (amplification primer)
RFP-nest_F: 5′- AAGCTGTCCTTCCCCGAGG GCTTC -3′ (sequencing primer)
RFP-nest_R: 5′- GGCCGCCGTCCTTCAGCTT CAG -3′ (sequencing primer)

Gene amplification conditions for primer pair 2-RFP_F/7-RFP_R and 42-RFP_F/7-RFP_R: reactions were performed in a final volume of 25 μl containing 20 ng of template DNA, 1X Promega Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM each dNTP (Promega), 10 pmol of each primer and 1 U of Taq Polymerase (Promega). Thermal cycling consisted of 30 cycles at 94°C for 30", 60°C for 30", and 72°C for 30". An initial denaturation step (94°C for 4') and a final extension holding (72°C for 7') were added to the first and last cycle. PCR products were analysed by 2% agarose gel electrophoresis using standard protocols, purified with the High Pure PCR Product Purification system (Roche) according to its protocol and then cycle sequenced for both strands with the nested sequencing-primers RFP-nest_F and RFP-nest_R using the BigDye Terminator Cycle Sequencing kit (both 62°C of melting temperature).

Results

About 150 larvae for each couple of breeders were obtained. The mortality rate was near to zero and fingerlings reached the adult size in three months. The 50% of F1 hybrids inherited the father's phenotype (i.e. the typical wildtype morphotype with black/blue longitudinal stripes), whereas the other 50% presented a hybrid morphotype with an atypical reddish-pink pigmentation of skeletal muscle analogous to that of female lineage and the blue-black stripped pigmentation of skin typical of wildtype males used. Any of the F1 hybrids have inherited the maternal phenotype only.

The amplification of both the 16S and Cytb genes confirmed all the individuals analysed were *D. rerio*.

Table I. List of referring sequences used in this work for species validation and RFP primer design for GM zebrafish. SC: synthetic constructs containing RFP sequences; CB: Cytochrome b fragments; 16S: 16S rDNA fragments; NCBI AN: NCBI accession number.

Type	Definition	NCBI AN
SC	Gateway binary vector pGWB455 DNA	*AB294467*
SC	Gateway binary vector R4pGWB553 DNA	*AB364194*
SC	Synthetic construct mCherry-hCdt1(30/120) gene for Fucci2-G1 phase probe	*AB512478*
SC	Gateway binary vector pGWB653 DNA	*AB543150*
SC	Gateway binary vector pGWB654 DNA	*AB543151*
SC	Gateway binary vector pGWB655 DNA	*AB543152*
SC	Gateway binary vector R4pGWB653 DNA	*AB543171*
SC	Mini-Tn7 delivery vector pUC18T-mini-Tn7T-Gm-DsRedExpress	*DQ493880*
SC	Mini-Tn7 delivery vector pUC18T-mini-Tn7T-Tp-DsRedExpress	*DQ493884*
SC	Mini-Tn7 delivery vector pUC18T-mini-Tn7T-Zeo-DsRedExpress	*DQ493888*
SC	Expression vector pDsRed2_ER red fluorescence protein DsRed2 gene	*EU016077*
SC	Synthetic construct isolate rNDV/F3aa/2seg dsRed protein (dsRed), M protein (M), F3aa protein (F3aa), and HN protein (HN) genes	*EU249348*
SC	Synthetic construct piggyBac mutator	*EU257621*
SC	Cloning vector L087 RRI-Red	*EU424173*
SC	Cloning vector pBGT1	*EU594544*
SC	Cloning vector pHC02	*FJ389159*
SC	Cloning vector pHC08	*FJ389162*
SC	Transient expression vector pX-DR	*FJ905211*
SC	Binary vector pCX-DR	*FJ905223*
SC	Cloning and transformation vector pHPdestmCherry	*GU142866*
SC	Expression vector pI2zCXCR4b6.6kbprom_mCherryF	*GU394077*
SC	Expression vector pTol2zCXCR4b250bp_prom_mCherryF	*GU394078*
SC	Expression vector pTol2zCXCR4bmutated250bp_prom_mCherryF	*GU394079*
SC	Expression vector pTol2zCXCR4b139bp_prom_mCherryF	*GU394080*
CB	*Danio rerio* cytochrome b gene, partial sequence	*EU241427*
CB	*Danio rerio* cytochrome b gene, partial sequence	*BC153924*
CB	*Danio rerio* cytochrome b gene, partial sequence	*BC122139*
CB	*Danio rerio* cytochrome b gene, partial sequence	*BC122143*
CB	*Danio rerio* cytochrome b gene, partial sequence	*AC024175*
CB	*Cyprinus carpio* cytochrome b gene, partial sequence	*HQ443697*
CB	*Cyprinus carpio* cytochrome b gene, partial sequence	*EU676848*
16S	*Danio rerio* 16S large subunit ribosomal RNA gene, partial sequence	*AY788011*
16S	*Danio rerio* 16S large subunit ribosomal RNA gene, partial sequence	*Z22694*
16S	*Danio rerio* 16S large subunit ribosomal RNA gene, partial sequence	*Z22695*
16S	*Danio rerio* 16S large subunit ribosomal RNA gene, partial sequence	*AF036006*
16S	*Danio rerio* 16S large subunit ribosomal RNA gene, partial sequence	*AC024175*
16S	*Cyprinus carpio* 16S large subunit ribosomal RNA gene, partial sequence	*NC001606*
16S	*Cyprinus carpio* 16S large subunit ribosomal RNA gene, partial sequence	*EU604688*

In particular, eight out of fifteen individuals shared the same 16S sequence (haplotype A), identical to the sequences retrieved in NCBI (Accession Numbers: AC024175, AF036006, AY788011, Z22694, Z22695), the remaining individuals' 16S haplotypes (haplotypes B, GenBank Accession Number JQ581533 - four out of 15 specimen; Haplotype C, JQ581534 - three out of 15) differed from them for only one substitution. Similarly, the two Cytb haplotypes we obtained (haplotype D, JQ581535 and haplotype E, JQ581536) differed for three substitutions from the published sequences (NCBI Accession Numbers: AC024175, BC1522143, BC152939, BC154924, EU241427).

The IFF gene amplification was performed to test the amplificability of nuclear DNA. All samples analysed gave the 220bp fragment as expected by Rehbein and Bogerd (2007). On the contrary, only two out of 15 suspected GMO and no hybrid F1 samples gave the expected 550bp product by using the 2206/2207 primer pair specific for red fluorescent protein detection. The related sequences together with those obtained by Rehbein and Bogerd (2007) and those of all possible homologous synthetic construct available at NCBI databank were aligned and used to design the new primers to detect RFP (Table I). The new primer pairs were designed to produce specific RFP amplicons of approximately 350bp (primer pair 2-RFP_F/7-RFP_R) and 280bp (primer pair 4-RFP_F/7-RFP_R). All 15 suspected GM *Danio* and the five F1 hybrids tested gave PCR product of the expected size, while any of the

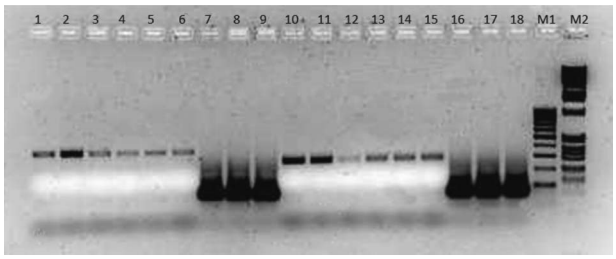

Figure 1. 2% agarose gel containing GelRed (Biotium) gel stain of RFP-positive zebrafish amplified using primers newly designed. All GMO samples and F1 hybrids showed a clear band of the expected size located in the RFP gene. Lines 1–9: primer pair 2_DsRed_F/7_DsRed_R (1–3: GMO 1–3; 4–6: HYB 1–3; 7–9: WILD 1–3), lines 10–18: primer pair 4_DsRed_F/7_DsRed_R (10–12: GMO 1–3; 13–15: HYB 1–3; 16–18: WILD 1–3). M1: 100bp DNA ladder; M2: 1kb DNA ladder (Invitrogen).

wildtype zebrafish tested as negative control gave PCR product (Figure 1). Similarly, all 20 RFP-positive samples cycle sequenced on both strands by primers RFP-nest_F and RFP-nest_R gave a clear electropherogram. Sequences obtained were quite similar to GenBank referring sequences number DQ493880, DQ493884, DQ493888, EU016077, EU249348, EU257621, EU594544, FJ905211 and FJ905223 (Table I) differing for a single mutation point (three out of five GM samples with a single G/A transition in position 151 and four out of five hybrid samples with an A/G transition in position 338), while differ from those of Rehbein and Bogered (2007) for two point mutations (C/T transition in position 164 and G/A transition in position 255). These mutations should have occurred by chance or should be ascribed to an error in PCR extension of fragments. In fact, even if the error rate of PCR enzyme is generally low, misincorporations should happen. The differences with all other RFP sequences available in GenBank are listed in Table I, ranging from 37 and 44 point mutations.

Discussion

The GM *Danio rerio* made via insertion of fluorescent protein with fast-muscle specific promoters were initially created for use in developmental and toxicological studies. The expression of fluorescent proteins in skeletal muscles gave those fishes a peculiar glowing colouration visible under normal light conditions (and emphasized under UV wavelengths) which resulted in them being appreciated by customers of the aquaria shops and they have begun to be commercialized under the trademark GloFish (http://www.glofish.com). While in other parts of the world like US or East Asian countries, engineered organisms are allowed, the commercialization and ownership of GMOs are strictly regulated by EU legislation (for an exhaustive review, see Plan & van den Eede 2010 and references therein). In particular, the two main legal instruments are (i) the Directive 2001/18/EC on the deliberate release into the environment of Genetically Modified Organisms, and (ii) the Regulation No 1829/2003/EC on genetically modified food and feed. The first one regulates the release of GMOs into the environment in the EU. The key objective of this Directive is to protect human health and the environment in relation to GMO release; it covers two types of GMO-related activities that is the voluntary or accidental release in the environment and the commercialization of GMO. In Italy this Directive was acknowledged by the Legislative Decree 224/2003. The second one provides the general framework for regulating GM food and feed in the EU.

As GloFish is a recognized genetic modification of the species *Danio rerio*, their commercialization is not allowed in the whole EU according to the above mentioned Directive. The intensification of inspections ordered in 2010 by the Italian Ministry of Environment and Territory (i.e. the competent authority on GMOs) has led to hundreds of confiscations and fines for many thousands of Euros. The same situation is reported by the GM Inspectorate of the Food and Environment Research Agency of the UK, GloFish division (a detailed list of incidents that have occurred in England starting from 2004 is available online at http://www.gm-inspectorate.gov.uk/gmfish/uktradeintropicalfish.cfm). For this reason it is of primary importance to have a molecular tool able to identify unequivocally GloFish, discriminating them from all other possible variants obtained by selective breeding, spontaneous mutations of skin colour or any other manipulation but not genetically modified (Parichy 2006).

Despite the three different morphotypes of samples analysed in this work, the mtDNA phylogenetic analysis showed that the 25 animals belonged unequivocally to *Danio rerio* species, excluding the possibility of the presence of different species or natural variants which should carry different skin colours (e.g. like the so-called "pearl danio" *Danio albolineatus*). This means that excluding the five wild-type morphotype, all other samples analysed belonged to an engineered variant and thus forbidden in Italy and other EU countries.

Primers designed by Ji and coauthors (2005) were developed with the precise purpose to amplify a very short fragment of the RFP gene to detect the copy number of RFP and therefore the zigosity of GM individuals in a TaqMan assay. The extreme

shortness of this amplicon (71 bp), which is useful for Ji's aim, is not suitable for an unambiguous identification of the PCR product by conventional PCR (and subsequent detection by agarose gel) and/or sequencing. Moreover the RFP primer set by Ji et al. (2005) was not designed for use in conserved sites and presents many single nucleotide differences in these regions among different RFP genes. For this reason Ji's primers do not amplify all RFP isoforms analysed in our paper.

In our experiment, only two out of 20 RFP-positive fish gave results with the 2206/2207 PCR test developed by Rehbein and Bogerd (2007). The RFP sequence alignment and related NJ tree topology allow us to discharge the hypothesis of presence of a different RFP variant and thus the failed amplification could be ascribed to point mutation in the target sequence for the 2206/2207 primer pairs.

Because of the criteria used here to design the new RFP detection primer pairs (only conservative sites of the alignment were considered), this new system not only showed better performances amplifying where other primer pairs failed, but also allowed the identification of GM fishes in which different variants of RFP are used. Moreover, since the shorter gene fragments are advantaged in amplification reactions, the reduced size of amplicons and the utilization of nested sequence primers, allow the utilization also in samples which had been subjected to severe DNA degradation due to bad preservation.

Even if the crossing experiment was performed in tanks, and therefore the choice of breeding couples was human-mediated, the crossbreeding experiment presented here seems to support the results of Snekser et al. (2006), who demonstrated that the red body colouration of transgenic zebrafish do not affect the shoaling behaviour nor the mating choice during night-time. The lack of preference for/avoidance of RFP transgenic zebrafish indicates that the overall body colour does not play a role in mating or social preferences in this species. The hypothesis of transmission of RFP gene also in F1 specimens suggested by the unusual skeletal muscle colouration presented by the 50% cross-hybrids obtained is confirmed by both PCR testing developed here and cycle-sequencing: all five hybrids analysed were positive to the presence of RFP showing the expected size amplicons and related DNA sequences were homologous to red fluorescent protein sequences. Although further studies on hybridization between GMO x wildtype danios would be necessary, the results here obtained imply that cross-hybrids obtained by female GM and wildtype *Danio rerio* males should also be included in the list of GMO regulated by Legislative Decree 224/2003.

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