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Fast Multiplex real time PCR method for sex-identification of medaka (*Oryzias latipes*) by non-invasive sampling

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A B S T R A C T

Medaka fish (*Oryzias latipes*) has been widely used in fish screening and multi-generation tests to provide relevant data to assess impacts of endocrine disrupting chemicals (EDCs) in fish populations.

The genotypic differentiation of Medaka sex allows diagnosing the sex reversal, and is required in current test guidelines (e.g. OECD TG 240, 2015). DNA isolation for genetic sex-identification requires sample collection, which has been normally conducted using invasive (fish sacrifice) or semi-invasive (fin-clip) procedures, which conflicts with the need for a fast, simple, and stress-free method. Swabbing skin mucus to collect DNA has been adopted in ecological studies of larger fish, however for smaller fish, it has to be established.

To handle larger number of samples, real-time PCR represents a faster and sensitive method compared to conventional PCR. In this study, we aimed to develop a multiplex real-time PCR method for Medaka genetic sex-identification, using DNA sampled by swabbing as less invasive technique. In this approach, the male-determining gene *DMY* was used in combination with the cytochrome *b* housekeeping gene.

- The method developed is a robust, rapid and a sensitive multiplex real-time PCR for Medaka genetic sex-identification.
- This method allows the use of DNA isolated from fish by swabbing, as non-invasive sampling method.

A R T I C L E I N F O

Method name: Multiplex real-time PCR

Keywords: Medaka, qPCR, Multiplexing, Non-invasive sampling

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Specifications Table

Subject Area:	***Biochemistry, Genetics and Molecular Biology Environmental Science***
More specific subject area:	*Molecular biology*
Method name:	*Multiplex real-time PCR*
Name and reference of original method:	*NA*
Resource availability:	*NA*

Method details

Protocol background

In ecotoxicological endocrine disruptor (ED) testing, the sex of exposed laboratory fish is generally determined by histopathological analyses of the gonads and classified as female, male, intersex or undifferentiated (phenotypic sex determination; [1,2]). Sex-identification using genetic tools provides the possibility to determine the sex of the fish independently from the phenotypic examination of the gonads, and thus in the living animal. This is of special importance if precise information on sex is needed during a running test, e.g. when setting spawning groups consisting of one male and female each (MEOGRT; [2,3]). When assessed in a regulatory context for ED testing, the sex ratio in treated compared to untreated fish groups can provide further indications for an endocrine mode of action of the tested substance. With Medaka as the test species, phenotypic sex reversal can be determined directly at the individual level.

In contrast to other commonly used small laboratory fish species (e.g. laboratory zebrafish (*Danio rerio*) strains), which lost their genetic sex-determining gene [4], sex in Medaka can be additionally determined genetically (genotypic sex determination). The Medaka fish has an XX/XY sex determination system, with the male-determining gene DMY being the first of this kind identified in non-mammalian vertebrates ([5]; reviewed by Matsuda and Sakaizumi [6], [12,13]). Thus, Medaka fish have been used as model for studying the mechanisms of sex determination, differentiation, and reversal. The XX/XY sex differentiation system in Medaka species refers to undifferentiated, i.e. homomorphic, sex chromosome. The term Y chromosome designated to a recombined chromosome that carries the male-determining gene *DMY*, a duplicate of the *dmrt1* gene, and X referring to the homologous chromosome without the *DMY* gene [5,13].

The *DMY* gene is conserved among all wild populations of *O. latipes* examined to date. The closely related species *O. curvinotus* also has *DMY* on its Y chromosome, which is orthologous to the *O. latipes* Y chromosome. However, *DMY* has not been detected in any other type of fish, including other *Oryzias* fish [5]. Therefore it is used in this method for the specific sex-identification of Medaka fish.

In Medaka, the female XX or male XY genes can be detected by Polymerase Chain-Reaction (PCR) [7,8]. However, for analyzing larger number of samples, real time PCR could represent a faster and more sensitive method than conventional PCR. Performing real time PCR is a sensitive technique, which requires high quality DNA, and for the presence/absence experiments it is particularly important to have an internal control or housekeeping gene to avoid false negative results. Combining the identification of the *DMY* (specific sex-identification) and the housekeeping cytochrome *b* (cyt b) gene [14] in one single reaction (multiplex PCR) allows the reliable genetic sex-identification of Medaka, using less sample in a faster and more economical way.

Furthermore, extracting DNA from fish samples for molecular analysis can be often invasive, or involve sacrificing the fish. Taking small fin clips is one of the non-invasive methods to collect DNA in fish, however, small laboratory fish especially suffer during this process or even end up dying, when the sample is taken by unexperienced staff. Swabbing skin mucus to collect DNA samples has been used in ecological studies of larger fish [9] and lately its use adopted for adult small laboratory fish species [10]. This technique potentially represents a less invasive alternative to fin clipping. There are several commercial kits available to isolate DNA from swabs, though there are some important

factors to consider when using this technique. The first one is the direction of the swabbing, due to the existence of scales; and the second one is the contamination risk with DNA from contact with other fish in close proximity (for example in high density tanks). However, studies have shown that even in high-density tanks there is no cross contamination, and that despite the lower extraction yields in comparison with what is obtained with fin clips, the DNA is sufficient for PCR analysis [11,10].

Sample collection and DNA extraction

For developing and optimizing the multiplexed real time PCR method, genomic DNA was isolated from Medaka fish (52 weeks old, 3–3.5 cm), where the sex was determined beforehand by visual inspection (anal fins with ossified papillary processes on the fin rays in male control fish). The samples were obtained using standard fin clipping procedures. A 2 mm large fin piece was punched out from non-anesthetized fish. The DNA was extracted using the First-DNA all-tissue Kit (GEN-IAL, Troisdorf, Germany), according the manufacturers' instructions.

Skin swabbing was used to collect DNA samples from the mucus of individual Medaka fish. For the different tests, old and young-in-age fish were used (52 weeks old, 3–3.5 cm; 10 weeks old, 1.25–2.5 cm). Skin mucus samples were collected by gently stroking with the tip of a sterile cotton swab (TecoMedical Mucus Collection Kit) on one side of the fish (following the orientation of the scales). The extraction of genomic DNA from the swab was conducted using the First-DNA all-tissue kit. The kit was applied according to the manufacturer's protocol with minor modifications. The swab was cut and the cotton part containing the sample was put in a 1.5 ml tube (Eppendorf) containing the lysis solution, and incubated for 30 min at 65 °C, with mixing at 800 rpm in a Thermomixer Comfort (Eppendorf). After incubation, the cotton swab was removed from the tube, squeezing its content against the tube walls before removal. The lysate was purified according to the standard protocol. The quality (260/280 ratio) and concentration of the extracted DNA was assessed by a nanometer (NanoDrop, ThermoScientific). A ratio between 1.8–2.2 was considered acceptable for qPCR analysis.

Multiplex amplification by real time PCR and melting curve analysis (Tm)

The amplification and melting curve analysis conducted by real time PCR (Light Cycler 96, Roche) using the SYBR Green approach, was selected to establish a multiplexing method targeting one Medaka sex-specific and Medaka housekeeping gene. The use of housekeeping genes in this technique is crucial in order to avoid false negatives in male determination. The specific primers were designed to target the *DMY* gene for sex-identification and the *cyt b* [14] as housekeeping gene. The master mix was prepared according the protocol described below.

Master mix preparation (stepwise description):

- Select the gene(s) to be amplified from Table 1.
- Prepare a master mix (DNA Green Master Mix (Roche)) for the number of samples to analyse, according to Table 2.
- Add 18 μL of the cocktail to each reaction tube
- Add 2 μL of DNA template
-

Table 1
Primer sets targeting the *DMY* and *cyt b* genes used in this protocol.

Target gene	Annealing temperature [°C]	Sequence (5′–3′)	Fragment size (bp)
DMY	55	F: TTG AAG ACC CCG CTG AAA G R: CGG CCC AAA TTC TGG CAT CTT TGC	355
cyt b	50	F: ACA ACT TCA CCC CTG CRA AY R: GAT GGC GTA GGC AAA TAG GA	80

Table 2
Multiplex qPCR Mastermix.

Reagent	Volume [μL]
DNA Mastermix kit, Roche	10
Water, Roche	6.5
DMY primer set (10 pmol/μL, Metabion)	0.5
cyt b primer set (10 pmol/μL, Metabion)	0.5
BSA (1 mg/mL, New England Biolabs)	0.5
Template	2

Run a PCR program according to the following conditions: initial DNA denaturation and enzyme activation step at 95 °C (10 min) followed by 30 cycles of denaturation at 95 °C (10 s), annealing at 62 °C (10 s), and extension at 72 °C (40 s).

- Run a final step for the melting curve analysis (Tm) consisting of: rapid heating of amplified samples to 95 °C to denature the DNA, cooling to 65 °C to let DNA strands anneal, slowly heating the samples to 97 °C, where the change in fluorescence is measured at each 0.1 °C rise for 1 s.
- Additionally, qPCR amplification products can be run using agarose gel electrophoresis to check for primer dimers and non-specific bands

Notes on the protocol:

1 For the development of the protocol, we worked with ROCHE Kits, but now we use the SensiFast SYBR No-Rox Kit from BIOLINE in our lab, providing the same results as with the ROCHE Kit.
2 The annealing temperature used in the described method is higher than the optimal temperatures described in Table 2 for each primer pair. This is in order to avoid primer dimer formation, which was observed during pre-tests and optimization stages.
3 When conducting PCR with one pair of primers only, the amount of primers was replaced by water.
4 A PCR product from a male-Medaka amplified using the *DMY* primers was purified (Cycle Pure Kit, Peqlab) and sequenced (Eurofins Genomics) for validating its identity.

Tm assessment by melting curve analysis after multiplexed real time PCR

The analysis is based on the different Tm values of the individual gene targets. In order to determine and identify the Tm patterns of each target gene, the genes were amplified in separated reactions using male and female control fish. An example of the dissociation curves patterns is shown in Fig. 1. As expected, only male Medaka fish DNA amplification is observed when targeting the DMY gene (Fig. 1a; Tm = 87.7 °C); and both, DNA from female and male control fish was amplified when targeting the cyt b gene (Fig. 1b; Tm = 80.5 °C). The amplified PCR product from male medaka using the *DMY* primers was sequenced. The sequences were confirmed to be those corresponding to *DMY* male medaka gene (data not shown).

When the real time PCR was run in the multiplex setup, the analysis of the Tm curves showed the same primer-specific patterns as the ones observed in the individual analysis (Fig. 2, compared to Fig. 1 a + b). Two peaks were observed in the samples containing DNA from male Medaka fish (at 80.5 °C and 87.7 °C corresponding to the specific amplification with the *cyt b* and *DMY* primers, respectively) and one peak for the DNA from female Medaka fish (80 °C for the *cyt b* primer).

Sex-determination with the developed method using swabbing for DNA extraction

To check the sensitivity and reproducibility of the developed method, DNA obtained from mucus collected from older male and female fish by swabbing (52 weeks old, 3–3.5 cm) was diluted (serial dilutions) to obtain concentrations ranging from 80 to 10 ng/μL.

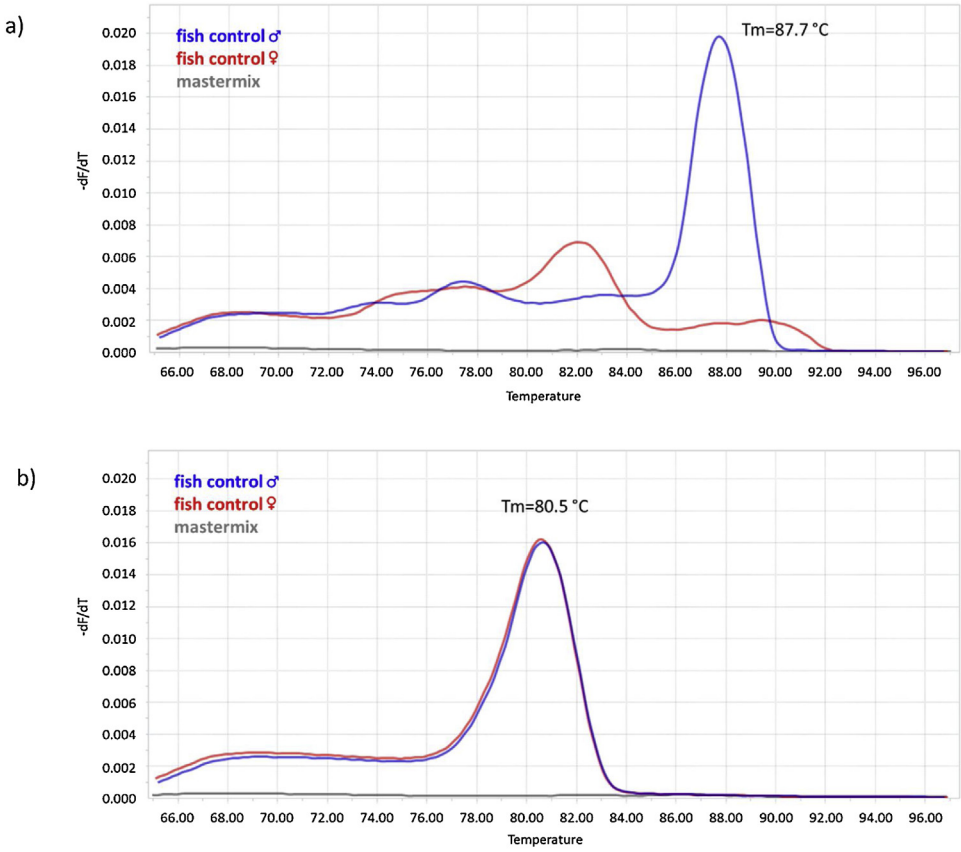

Fig. 1. Tm patterns of control fish amplified with a) *DMY*; b) *cyt b* primers.

Normally, it is possible to isolate more DNA by swabbing larger Medaka fish than from smaller fish (i.e. young-in-age fish), however with the developed method it is possible to obtain reliable DNA amplification even at low DNA concentrations. To test this, we also isolated DNA from 10 Medaka fish (10 weeks old, 1.5–2 cm, sex not yet differentiated) using swabbing of mucus for DNA collection.

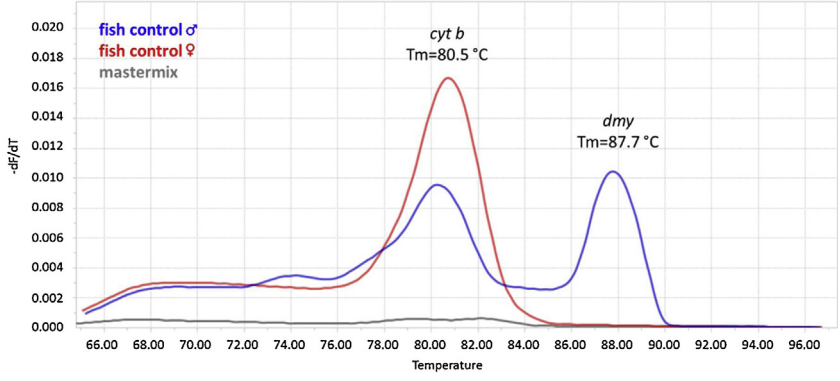

Fig. 2. Dissociation curves patterns for the multiplex real-time PCR method.

Table 3
Results of the melting curve analysis at different DNA concentrations (serial dilution) obtained from previously identified male and female Medaka.

Type of sample	DNA concentration [ng/μl]	Tm 1 (*DMY*)	Tm 2 (*cyt b*)
Medaka male	72	87.69	80.30
	61.4	87.74	80.35
	41.7	87.77	80.37
	24.6	87.71	80.33
	12.1	87.64	80.45
Medaka female	82.8	–	80.89
	62.2	–	80.72
	44.7	–	80.58
	18.9	–	80.56
	8.6	–	80.55

Table 4
Sex-identification of young-in-age Medaka fish using the developed multiplexed real time PCR method in comparison to traditional visual inspection of the fish.

Sample	[ng/μL]	TM1	TM2	Result PCR	Result visual inspection
Unknown fish 1	17.6	87.77	80.64	♂	♂
Unknown fish 2	8.3	87.97	80.81	♂	♂
Unknown fish 3	6.2	–	81.00	♀	♀
Unknown fish 4	20.8	–	80.88	♀	♀
Unknown fish 5	38.4	–	80.87	♀	♀
Unknown fish 6	36.2	–	81.09	♀	♀
Unknown fish 7	14.2	87.95	80.84	♂	♂
Unknown fish 8	5.6	–	81.14	♀	♀
Unknown fish 9	10.2	–	80.98	♀	♀
Unknown fish 10	10.6	–	81.14	♀	♀

The results showed that it is possible to identify the sex of Medaka in the selected concentration range (serial dilution, Table 3), even to a minimum concentration of 8.6 ng/μL. Also, if DNA was extracted from the young-in-age fish (Table 4), identifying reproducible peak patterns for both sets of primers used (Fig. 3) was possible. In both cases, results were validated by visual inspection of the fish.

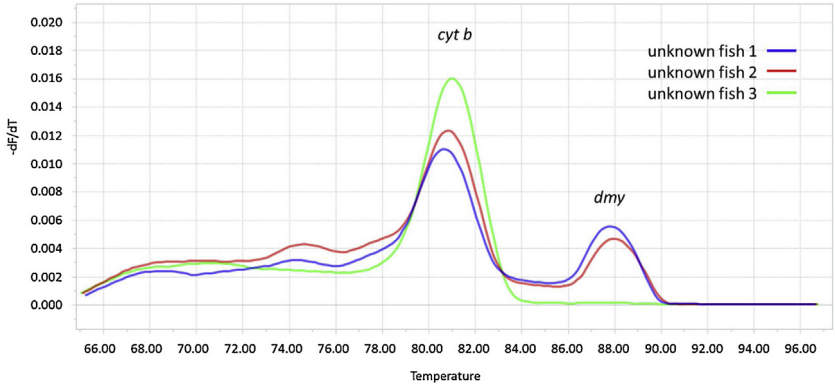

Fig. 3. Dissociation curves of young-in-age Medaka fish.

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