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A new sex-specific genetic marker (*fshr* 1834G>T) for flathead grey mullet, *Mugil cephalus*, in Queensland, Australia

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ABSTRACT

In this study, genetic sex marker candidates from northern hemisphere *Mugil cephalus* were tested to see if they could be used to sex a population originating from south-east Queensland, Australia. As such, a region of the follicle stimulating hormone receptor (*fshr*) gene was sequenced but did not contain previously published single nucleotide polymorphisms (SNPs). However, further screening of the sequenced *fshr* region revealed a promising sex marker candidate for Queensland *M. cephalus*, *fshr* 1834 G>T, which was accurate in 100% of fish tested (excluding intersex fish, which had the female genotype). While all females tested were homozygous G/G, males presented as either G/T (common) or T/T (lower frequency). Subsequently, a real-time high-resolution melt was developed to facilitate rapid and accurate genotyping of *M. cephalus* based on the *fshr* 1834 G>T SNP. Initial results suggest that *fshr* 1834 G>T is a useful SNP that can reduce the need for more invasive sampling techniques such as gonadal biopsy, provide information relating to the sex of captive stock prior to gonadal maturation, and may prove useful in wild population surveys and stock assessment.

1. Introduction

The flathead grey mullet (*Mugil cephalus*) is a circumglobally distributed catadromous species, inhabiting rivers, lagoons, estuaries, and inshore marine environments between approximately 42°N - 42°S (Crosetti et al., 1994). Mugil cephalus has socioeconomic importance as a wild fishery and aquaculture species, with potential in aquaculture and ecosystem bioremediation (Saleh, 2008; Whitfield et al., 2012; Shpigel et al., 2016; Biswas et al., 2020; Liu et al., 2021). Expansion of *M. cephalus* aquaculture therefore enables the production of fish for improved food and nutritional security (flesh and roe), environmental outcomes, and stock enhancement programs.

Central to successfully breeding *M. cephalus* is the optimisation of maturation and spawning conditions, including sex ratio, which requires accurate identification of sex (Aizen et al., 2005; Besbes et al., 2020). In addition, field-based studies on demographic structure also require methods of sexing mature and immature fish where samples can be collected rapidly using minimally invasive techniques. As *M. cephalus* does not exhibit external sexual dimorphism, biotechnological approaches have been developed to sex fish, including plasma vitellogenin dot-blot during sexual development (Aizen et al., 2005), and polymorphism-based sex markers in the follicle stimulating hormone receptor (*fshr*) gene for mature and immature fish (Curzon et al., 2021; Ferraresso et al., 2021). Since published genetic sex markers have never been tested on Australian mullet, the aim of the current study was to determine whether existing *fshr* sex markers could be utilised for a Queensland (Australia) population of *M. cephalus* and to examine a partial segment of the *fshr* gene to find other candidate sex markers if needed. For efficient sexing of fish, a real-time high-resolution melt (RT-HRM) assay targeting a promising sex-specific single nucleotide polymorphism (SNP) was subsequently developed.

2. Methods

2.1. Animal ethics statement

Animal capture, maintenance, and sampling were conducted under the Department of Agriculture and Fisheries (DAF) General Fisheries Permits (186281 and 213514) and Animal Ethics Committee Approval (CA 2020–04–1366).

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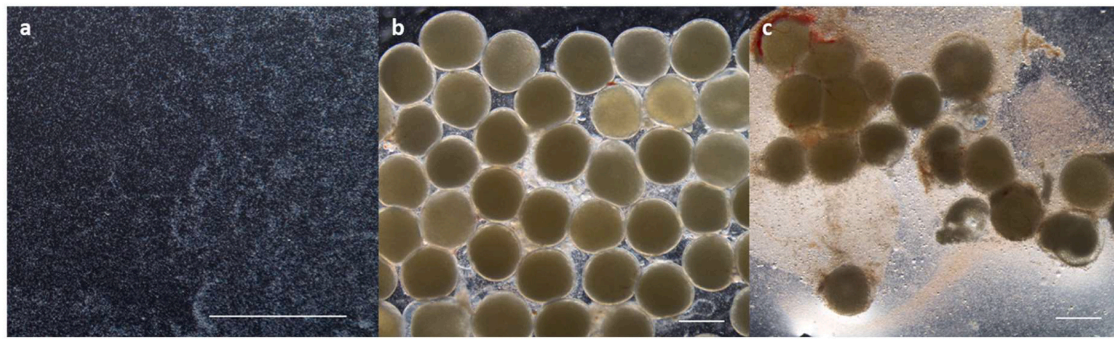

Fig. 1. Gametes of a representative phenotypic (a) male, (b) female, and (c) intersex *M. cephalus*. Scale bars = 500 μm.

2.2. Fish capture and sampling

M. cephalus belongs to a species complex that is yet to be fully resolved (Reviewed by Whitfield and Durand, 2023). The cytochrome oxidase subunit I (COI) gene sequence for *M. cephalus* collected from North Pine River in Queensland (proximate to the collection locations in this study) is available on GenBank (HM006970, Page and Hughes, 2010). When compared to COI sequences from *M. cephalus* caught in other regions, this sequence has 99.17% (northwest Pacific, GU260680), 99% (New Zealand, KX639486), 99% (Philippines, OQ386362), 99% (Turkey, KC500933), 98.84% (India, KT347598), 98.34% (Egypt, LC490615) and 98.34% (Israel, KY683176) homology. For the purposes of this study, fish with the morphological features listed on FishBase.org (Froese and Pauly, 2023) were considered to be M. cephalus (Fig. S1).

Two cohorts of adult *M. cephalus* were collected for aquaculture research programs located at Department of Agriculture and Fisheries (DAF) Bribie Island Research Centre (BIRC), Woorim, Queensland, Australia. Cohort 1 (n = 4) was acquired from commercial fishers using beach seine netting at Skirmish Point, Queensland (−27.089043°S, 153.206362°E) in June 2020 during their spawning migration. Cohort 2 (n = 20) was collected using a boat-mounted electrofisher from South Pine River, Queensland (−27.304076°S, 152.997191°E) prior to spawning migration in March 2022. Fish were transported by road in a 600 L oxygenated fibreglass carrier and quarantined, acclimated, and maintained in 7000 L aerated fibreglass tanks supplied by flow through seawater. Fish were implanted with passive integrated transponder tags (Trovan®, Germany) before cohorts were combined. Fish were maintained under natural photoperiod supplemented with artificial lighting and fed ad libitum twice per day with a combination of commercial prawn pellet (Ridley Aquafeeds Ltd., Australia) and pelletised feed formulated at BIRC.

For all fish, gonadal biopsies were performed at multiple time points during gonadal maturation to confirm phenotypic sex. All samples were viewed under a stereomicroscope, and where appropriate, sperm was activated with a drop of sea water.

Fin clips were collected from all fish and stored in 100% molecular grade EtOH at − 20 °C. DNA was then extracted using the Bioline ISOLATE II Genomic DNA Kit (Meridian Bioscience®) and quantified using a NanoDrop-1000 (ThermoFisher Scientific, Inc.).

2.3. Genetic marker identification

The SNP containing region of *fshr* described by Curzon et al. (2021) and Ferraresso et al. (2021) for fish from the northern hemisphere (Israeli Mediterranean coast, Mediterranean sea, and Spain) was targeted for investigation. All *M. cephalus* DNA/cDNA sequences available on GenBank (National Center for Biotechnology Information) for male and female fish were aligned using Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/ (LR860656.1, LR862571.1, LR860660.1, LR860659.1, LR860657.1, XM_047608127.1, and LR860658.1), and primers were designed using Primer3 (https://bioinfo.ut.ee/primer3–0.4.0/) to amplify a 452 bp fragment containing the most promising published SNP locations. The forward (5′−3′ GCATCTGCTTGCCCATGGAT) and reverse (5′−3′ TGCTGGCTGCTGACAGGAAG) primers had an annealing temperature of 60 °C.

Each PCR reaction contained: 1 μl DNA (diluted 1:50), 500 nM each primer, 1 μl 10 x buffer (Qiagen, Cat. No. 201203, includes 25 mM $MgCl_2^+$), 250 μm each dNTP, 0.1 μl Taq DNA polymerase (Qiagen, Cat. No. 201203), and molecular grade water to a final volume of 10 μl. PCRs were run on a T100 thermal cycler (Bio-Rad Laboratories, Inc.) with the following conditions: 95 °C for 2 min; 50 °C for 45 s; 72 °C for 1.5 min; 34 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C 1.5 min; and then a single final step of 72 °C for 7 min

PCR product size was confirmed via agarose gel electrophoresis, and then amplicons were cleaned using ExoSAP-IT (Thermo Fisher Scientific, Inc.) and sequenced in the forward and reverse direction via Sanger sequencing at the Australian Genome Research Facility (AGRF, St Lucia, Brisbane). Traces were visualised using Unipro UGENE v37, and trimmed sequences were aligned with each other and published *fshr* sequences for SNP identification. The sequences for males and females were translated using Expasy (https://web.expasy.org/translate/).

2.4. RT-HRM development

A real-time HRM assay was developed around a single base mutation (1834 G>T) in *fshr*. To guide primer design, melt temperatures of the amplified products were predicted using uMELT Quartz (Dwight et al., 2011). The best-performing assay anchored the allele-specific primers downstream of 1834 G>T (5′−3′ shared forward: CGGCCGCGCTCAAGAC, 'X′-specific reverse: *gtttctt*GGAGGACCTTAGATTCCGAG<u>G</u>C, and 'Y′-specific reverse: GGAGGACCTTAGATTCCGAG<u>C</u>A). The specificity of the allele-specific primers was increased by placing a mismatched nucleotide in the 3′ penultimate position (underlined bases) (Cha et al., 1992). To improve melt curve separation, additional bases (lower case bases) were added to the 5′ end of the 'X′-specific primer. The 'X′-specific assay produced a 58 bp product, while the 'Y′ assay product size was 51 bp.

Reactions consisted of 2 μl of DNA (diluted 1 in 50), 0.33 μM of each primer, 7.5 μl of Type-it HRM (Qiagen, containing reference dye EvaGreen) master mix, and water to a total volume of 15 μl. Cycling conditions were: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 53 °C for 30 s, and 69 °C 10 s (acquire); melt: 75 °C for 2 s, increment of 0.15 °C/s with acquire, followed by 90 °C for 2 s. The Applied Biosystems™ qPCR Analysis Software High Resolution Melt module (Thermo Fisher Scientific) was then used to generate melt curves and difference plots.

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             1689C>A                                    1732G>A
Ref male     CATTCCGCAATCCCTCTTCGGTGCCGGCCCACGCCGACACGCGCATGGCTCAACGCATGG

Ref female   CCTTCCGCAATCCCTCTTCGGTGCCGGCCCACGCCGACACGCGCGTGGCTCAACGCATGG

Qld male     CCTTCCGCAATCCCTCTTCGGTGCCGGCCCACGCCGACACGCGCGTGGCTCAACGCATGG

Qld female   CCTTCCGCAATCCCTCTTCGGTGCCGGCCCACGCCGACACGCGCGTGGCTCAACGCATGG
             * ****************************************** ***************
                    1759T>G
Ref male     CCGTCCTCATCGTCACCGACTTCATCTGCATGGCCCCGATCTCCTTCTTCGCCGTCTCGG

Ref female   CCGTCCTCATCTTCACCGACTTCATCTGCATGGCCCCGATCTCCTTCTTCGCCGTCTCGG

Qld male     CCGTCCTCATCTTCACCGACTTCATCTGCATGGCCCCGATCTCCTTCTTCGCCGTCTCGG

Qld female   CCGTCCTCATCTTCACCGACTTCATCTGCATGGCCCCGATCTCCTTCTTCGCCGTCTCGG
             *********** ************************************************
                                     1834G>T
Ref male     CCGCGCTCAAGACCCCCCTCATCACCGTCTCGGAATCTAAGGTCCTCCTGGTCCTGTTCT

Ref female   CCGCGCTCAAGACCCCCCTCATCACCGTCTCGGAATCTAAGGTCCTCCTGGTCCTGTTCT

Qld male     CCGCGCTCAAGACCCCCCTCATCACCTTCTCGGAATCTAAGGTCCTCCTGGTCCTGTTCT

Qld female   CCGCGCTCAAGACCCCCCTCATCACCGTCTCGGAATCTAAGGTCCTCCTGGTCCTGTTCT
             ************************** *********************************
                  1875C>T                                     1917C>T
Ref male     ACCCCATTAACTCGTGCGCCAACCCCTTCCTGTACGCCTTCTTCACCCGCACCTTCCGGC

Ref female   ACCCCATCAACTCGTGCGCCAACCCCTTCCTGTACGCCTTCTTCACCCGCACCTTCCGGC

Qld male     ACCCCATCAACTCGTGCGCCAACCCCTTCCTGTACGCCTTCTTCACCCGTACCTTCCGGC

Qld female   ACCCCATCAACTCGTGCGCCAACCCCTTCCTGTACGCCTTCTTCACCCGTACCTTCCGGC
             ******* ***************************************** **********
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Fig. 2. Alignment between previously published *fshr* reference (Ref, GenBank LR860658.1 and LR862571.1) and Queensland consensus (OR236463 and OR236464) sequences for male and female *M. cephalus*.

3. Results and discussion

3.1. Phenotypic sex

The phenotypic makeup of the 24 fish sampled included 8 males (activated sperm), 14 females (oocytes) and 2 intersex (oocytes and activated sperm) (Fig. 1). Intersex in *M. cephalus* has been previously observed in several studies; for example, in wild populations from the east coast of India (Dhanasekar et al., 2018) and in extensively farmed fish from a brackish lagoon in Sardinia, Italy (Antuofermo et al., 2017).

3.2. A comparison of northern hemisphere and Queensland *fshr* sequences

The previously published reference sequences (LR860658.1 and LR862571.1) and Queensland consensus sequences (OR236463 and OR236464) for *M. cephalus* were aligned. The polymorphisms 1689 C>A, 1732 G>A, 1759 T > G (*fshry* haplotype), and 1875 C>T (Curzon et al., 2021) were not present in the Queensland *M. cephalus* sampled and all Queensland fish contained the female-type SNP regardless of sex (Fig. 2). Therefore, sex-biased SNPs from northern hemisphere populations of *M. cephalus* cannot be used to determine sex for the Queensland population studied.

A 1917 C>T polymorphism was present when comparing the reference and Queensland fish (Fig. 2). In terms of sequences available on GenBank, the T present in Queensland-caught *M. cephalus* was also present in one sequence from a juvenile of unknown sex caught in India (XM_047608127.1, data not shown).

3.3. Incidence of a newly characterised sex-specific SNP

A new sex-specific candidate SNP (1834 G>T) was identified, that was not present in northern hemisphere fish (Fig. 2). Of the Queensland *M. cephalus* sequenced, all 14 females were homozygous G/G, 2 intersex fish were homozygous G/G, 7/8 sequenced males were heterozygous G/T, and 1 male was homozygous T/T. Thus, both intersex fish aligned with the female genotype. The female codon (GTC) would result in the synthesis of valine, heterozygous males (GTC and TTC) encode valine and phenylalanine, respectively, while the homozygous male (TTC) encodes only phenylalanine. Similarly, the *fshr* SNP (1759 T > G, *fshry* haplotype) in a northern hemisphere population of *M. cephalus* results in the substitution of phenylalanine for valine, altering a conserved position in the transmembrane domain which may impact Fsh signalling (Curzon et al., 2021). The single homozygous T/T male has expressed milt without the need for hormonal intervention and does not appear to display reproductive dysfunction.

Genetic sex determination in *M. cephalus* is complex, no evidence for the presence of heterochromatic sex chromosomes has been found, and the accuracy of *fshr* sex-markers typically vary between populations (Ferraresso et al., 2021). While all female and male fish were sexed correctly using 1834 G>T in this study, testing of this marker on a larger population of fish and on fish from different regions would enable a more robust assessment of its accuracy and applicability.

3.4. RT-HRM performance for 1834 G>T

Using the automatic cycle threshold of 8446, the sample reaction cycle threshold scores (Ct scores) of the RT-HRM assay ranged between

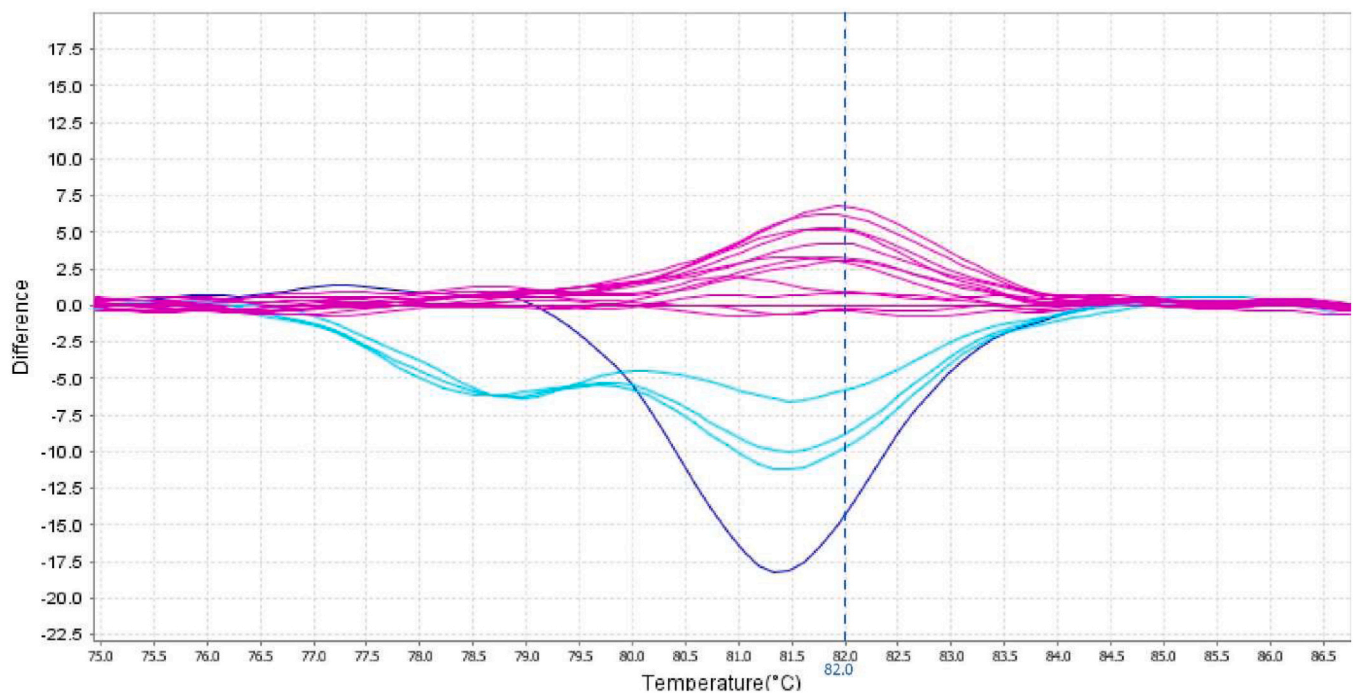

Fig. 3. Difference plot for the optimised RT-HRM mullet sexing assay. Females/intersex are coloured magenta (G/G), and males are coloured teal (G/T) or dark blue (T/T).

24 and 26. Amplified product melt temperatures for the different genotypes were 81.51 °C for G/G females/intersex, 81.22 °C for G/T males, and 80.93 °C for the T/T male. The difference in product melt temperature between G/G and G/T fish was 0.3 °C, G/G and T/T was 0.59 °C, and G/T and T/T was 0.3 °C. The difference plot of the RT-HRM assay clearly differentiates between female/intersex and male fish (Fig. 3). The difference plot of heterozygous males is characterised by a double melt curve and is distinct from the homozygous male (Fig. 3).

4. Conclusion

The genetic sex marker candidates available for northern hemisphere *M. cephalus* were not suitable for the Queensland population studied. A promising sex marker candidate for Queensland *M. cephalus* has been identified, *fshr* 1834 G>T, which was accurate in 100% of fish tested (excluding intersex fish which had the female-type SNP). In addition, males presented with two genotypes (G/T and T/T), and the lower frequency T/T genotype fish appears to display normal reproductive function in aquaculture conditions. Testing of *fshr* 1834 G>T on a larger number of samples and in fish from different regions will provide more detailed information about its broader reliability and applicability given that *M. cephalus* is a species complex and this SNP was not present in fish from Israel and Spain. A RT-HRM genetic assay was developed to facilitate rapid and accurate testing. These initial results suggest that *fshr* 1834 G>T is a useful SNP that can reduce the need for more invasive sampling such as gonadal biopsy, provide information relating to the sex and genotype of captive stock prior to gonadal maturation. This approach may also prove useful in wild population surveys and stock assessment.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101858.

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