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A RAPID PCR ASSAY FOR IDENTIFICATION OF MALES IN WIDOW TETRA, *GYMNOCORYMBUS TERNETZI* (BOULENGER, 1895)

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ABSTRACT
Non-invasive, early sex determination using molecular markers have potential application in aquaculture and human genetics. Double Sex/Male abnormal Protein (mab3) related transcription factors (*Dmrt*) family genes may be useful as Y chromosome-specific markers for determining maleness in fishes and humans. **Objectives:** To develop a rapid PCR assay for early identification of androgenetic clones (Y^2Y^2) and unexpected females (X^1Y^2) with male genotype in widow tetra (WT), *Gymnocorymbus ternetzi* larvae using *Dmrt1*, a male-specific marker. **Methods:** Genomic DNA isolated from larval tail fins of 8 androgenetic males (Y^2Y^2) and 4 unexpected female progenies (X^1Y^2), produced by progeny testing of androgenetic males were selected. A robust PCR protocol was optimized using primers for *Dmrt1* specific genes (*Dmrt1*F –AAGTGCTCCCGCTGCCGGAA; *Dmrt1*R -GCTGGTACTGCTGGTAGTTG). The amplified genomic products were identified using agarose gel (2.5%) electrophoresis, cloned and sequenced. **Results:** *Dmrt1* specific primer amplified a Y chromosome-specific 300 bp and 237 bp amplicons in WT males which was absent in females. Sequencing of the 237 bp amplicon confirmed homology (84%) with previously reported medaka and Buenos Aires tetra (BA tetra) *Dmrt* sequences. **Conclusion:** Y chromosome-specific markers have profound applications in human foetal sex determination and early identification of males in genetically manipulated fishes.

KEYWORDS: Dmrt1, Y chromosome-specific markers, PCR assay, genomic DNA, androgenetic clones, Human.

INTRODUCTION
Molecular mechanisms responsible for sex determination remain diverse from insects, fishes to advanced endocrine control of sex in mammals [1]. Teleosts exhibit sexual dimorphism and species-specific sex determination mechanisms ranging from genetic sex (XX/XY/ ZZ/ZW; [2], temperature dependence [3] to protogynous or protoandrous Sex determination[4], that change sex with advancing years and growth. Recently, *Dmrt* (Double Sex Mab3 related Transcription factors) family were shown to be prominently involved in sex determination and differentiation from invertebrates to vertebrates, gaining considerable importance among diversified teleost species [4]. Recent studies confirmed that DNA binding domain of *Dmrt* genes shared a common motif between all its homologues in different species and their ancestral origin and phylogenetic relationships with lower organisms were determined [5]. Being tissue-specific developmental regulators, *Dmrt1* are invariably expressed at different stages of embryonic division, gonadal development and temperature dependant gonadal sex-reversal [4, 8-12]. As *Dmrt* gene resides in the Y chromosome and remains conserved across many species including birds [8], reptiles [9], humans [10, 11] and fishes [11-14] and their ability to be a reliable potential biomarker for male-sex-specific determination is unquestionable.

Our understanding of sex determination and differentiation mechanisms in fishes is at its infancy. Several novel bio-markers have been characterized such as sex chromosome-specific repetitive sequence in humans [8, 10, 11], poecilids [2], sex-specific quantitative DNA markers in *Onchorrynchus tshawytscha* [15], sex-determining region Y (SRY) in the rosy barb, *Puntius conchonius* [16] and *Dmrt* in Medaka, *Oryzias latipes* [6, 7], Buenos Aires (BA) Tetra, *Hemigrammus caudovittatus* [13, 14], zebrafish, *Danio rerio* [17]and also tilapia, *Oreochromis mossambicus* [3], which undergoes both genetic and temperature dependant sex determination. Obviously, *Dmrt* family of transcription factors is the most abundant sex-specific marker available with several homologues (i.e. *Dmrt1a*, *Dmrt1b*, *Dmrt2*, *Dmrt3 and Dmrt4* up to *Dmrt7*) [2, 8, 10]. Our earlier studies have confirmed its genetic and hetero-chronic expression pattern as a potential male sex determining gene in *O.*

latipes[12] and *H. caudovittatus*[13,14]. As master regulators, its regulatory role for maleness is confirmed only based on position-specific expression in embryos and larvae redirecting certain subset of cell population i.e. primordial germ cells (PGCs) to differentiate into testis in males but functional role of *Dmrt* in genetic sex determination yet remains to be completely understood [4].

Tetras have recently become an attractive genetic model owing to their small size, cost and ease of maintenance in less space, oviparous nature, external fertilization with transparent embryos and larvae, visceral organs with structural, functional and genomic similarity to humans [13, 14, 18, 20, 21]. Previously, we confirmed paternity of *H. caudovittatus* androgenotes (Y^2Y^2) based on phenotypic (body and fin pigmentation), cytological markers (RBC diameter and karyotyping) and *Dmrt1* sex-specific molecular marker [13, 14]. Similar to the *H. caudovittatus*, when androgenesis was induced in *G. ternetzi* with fresh [19] and cryopreserved sperm [20], androgenotes suffered delayed sexual maturity (6 months). Progeny testing of WT androgenetic males also produced un-expected female progenies (X^1Y^2) with male genotype, instead of the expected 100% male sex ratio (data not shown) hence early identification of males was required. In the present study, our objectives were to develop a robust PCR based assay using *Dmrt1* Y chromosome-specific markers for (i) early identification of control and androgenetic WT males generated using fresh and cryopreserved sperm and (i) to confirm genotype of unexpected females (X^1Y^2) produced during progeny testing of androgenetic males [20].

MATERIAL AND METHODS

Isolation of genomic DNA

For DNA extraction, caudal fin clippings (non-invasive; <10mg each) of larvae were collected from desired individuals. Non-invasive caudal fin clipping did not harm the individual as the fin regenerated within ~40 days. DNA was extracted, quantified using spectrophotometer (Eppendorf, USA) and good quality genomic DNA were used for the experiments following previously established protocols ([13, 14, 18, 21].

PCR analysis

For the study, a set of primers were designed, for amplifying Y chromosome-specific molecular markers for the fish DMRT1F (AAGTGCTCCCGCTGCCGGAA) and DMRT1R (GCTGGTACTGCTGGTAGTTG) based on conserved sequences published for DMRT in *O. latipes* (Accession No. AY442916) [2, 7] and BA tetra (Accession no: AB091696) [13] from Pubmed library of sequences,. Using these primers, genomes (100 ng each) of control males (X^1Y^2) androgenetic males (Y^2Y^2), unexpected females (X^1Y^2) of WT were amplified in Thermal cycler (ABI, USA). The PCR protocol was as follows: denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec and primer extension at 72ºC for 40 sec. Thirty cycles of reaction were performed with a final extension at 72ºC for 5 min and initial denaturation at 94ºC for 3 min. The resulting PCR products were analysed on a 2.5% agarose gel and imaged using a Gel documentation system (Biorad, USA).

Cloning and sequencing

PCR amplified products were eluted from the agarose gel for cloning and sequencing [22]. Briefly, amplified PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA) and recombinant clones selected and sequenced with universal and custom primers, following Sanger's di-deoxy termination method in an automated DNA sequencer (ABI Prism 377, USA) using Big-dye terminator sequencing kit (Perkin- Elmer). The nucleotide sequence was analysed using GCG software in a Unix-based server following established protocols [13].

RESULTS

PCR analyses

Genomic DNA from larval tail fins were amplified with *Dmrt1* primers. Two amplicons (237 bp and 300bp) were amplified in the genomic DNA of males (Figure 1). The consistent presence of the 2 amplicons in male genome and absence in females confirmed that amplicons were indeed male sex-specific and specific to Y chromosome of the WT. The earliest time where *Dmrt1* can be identified during embryogenesis is 24hpf; constantly expressed thereafter in larvae up to seven days post hatching (dph) confirming its ability to determine sex as early as 24hpf – 7dph, in tetras instead of the long wait for 4 months to reach sexual maturity for progeny testing.

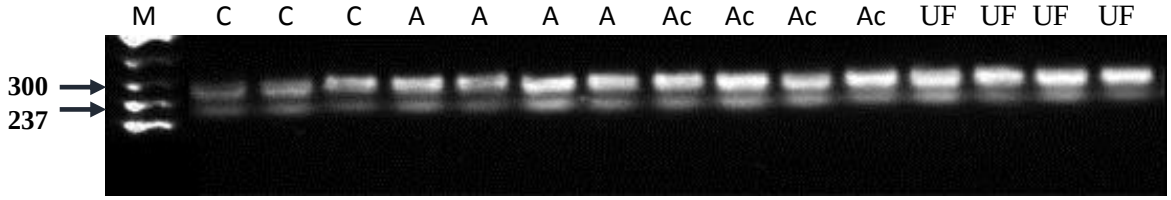

Figure 1. PCR products amplified by *Dmrt1* specific primers in genomic DNA of Control males (C), Androgenetic males generated using fresh sperm (A), Androgenetic males generated using cryopreserved sperm (Ac) and Unexpected female progenies (UF) with male genotype generated during progeny testing of Androgenetic males (Ac). M- 100bp marker.

Cloning and sequencing

PCR amplified 237 bp amplicon was cloned into pGEMT vector and selected based on blue white colony selection to determine clones carrying the *Dmrt1* product for sequencing. Sequencing of the 237bp amplicon confirmed its homology (84%) with the existing *Dmrt1* sequence reported from *H. caudovittatus* ([13, 14] *and O. latipes* ([2]. Expectedly, *Dmrt1* amplified only in genomes of control males, androgenetic males and unexpected females with male genotype but not in females (X^1X^2), thus confirming that *Dmrt* sequence is bound to the Y chromosome of *G. ternetzi* (Fig. 1). However, *Dmrt1* amplification in females with male genotype indicates genotype-specific ($Y^2Y^2/X^1Y^2/X^2Y^2$) species identification is also possible, thus confirming its authenticity as the Y chromosome-specific molecular marker in tetras and other vertebrates.

DISCUSSION

Sex determination and differentiation in fishes are complex mechanisms that involve genes, temperature, sex-specific markers, endocrinal and family of transcription factors i.e. *Dmrt* and *SOX9* (reviewed in [4]). *Dmrt* family (double sex) was first reported in *Drosophila melanogaster,* as a mutation affecting sex determination during genetic screening hence, thought to be primarily confined to fly genome [23] but their alternative splicing driving sex determination from insects to vertebrates were confirmed later. Despite profound differences in mechanism of sex determination from drosophila to mammals, it has been proven that *Dmrt* family members were present in every animal lineage by evolutionary and phylogenetic analyses [4]. The primary functions of *Dmrt* genes in fishes were to promote male gonad development and repress female specific differentiation, similar to humans; expressed invariably in gonad determining cell lineages in most vertebrates including mammals [8, 9, 11], birds [8], reptiles [9], amphibians [24] and teleosts [2, 4, 6, 7, 12-14]. In turtles, where sex is determined by ambient temperature, *Dmrt1* expression was significantly up regulated in male gonads preceding sexual differentiation [9]. Due to teleost specific genome duplication event (TSD), several homologues of *Dmrt* genes were reported from *O. latipes* [12, 7]. Further, *Dmrt1* expression precedes testes development in rainbow trout [25 and exclusively expressed in testicular tissues of several fishes and confirming their role in sex-determination and differentiation [26].

In Aquaculture, where genome manipulation techniques are used to generate androgenetic diploids, triploids or tetraploids for increasing body weight and growth mostly, males remain fertile hence, early identification of sexes in ploidy manipulated fishes may be beneficial to aquaculture [1]. Confirmation of paternity in androgenotes were mostly through phenotype (body and tail fin pigmentation) and karyotype since, sex-chromosomes in fishes remain morphologically indistinguishable, sex-chromosome-specific molecular markers for differentiating males from females were required. In androgenotes, carrying single (haploid) or double (diploid) copies of exclusively paternal genome alone, application of *Dmrt1* as male-specific markers has been fruitful in *H. caudovittatus* [13, 14], *O. latipes* [12] and *G. ternetzi* (present study). Progeny testing of androgenetic males known to result in 100% male population is a desirable trait and a common practice in aquaculture for genetic improvement of strains but requires identification of males by manual sexing after sexual maturity [1]. However, crosses involving androgenetic *G. ternetzi* males (Y^2Y^2) and control females (X^1X^2) resulted in unexpected females (X^1Y^2) [20]. Further, triploids [18] and tetraploid males of tetras [21] that may provide diploid gametes for generation of triploids up to hexaploids without requiring artificial manipulation techniques also require sex-specific markers to facilitate early identification. From the present study, we confirm that *Dmrt1* can be used as a reliable male-specific and Y chromosome-specific marker for identifying males in tetras [13, 14, 20]. However, RT-PCR analyses to detect their temporal and spatial expression pattern in gonadal and somatic tissues and consequences of multiple genome copies on sex-determination in triploids [18] and tetraploids [21], may be shed light on sex differentiation mechanisms in fishes.

In humans, haplo-insufficiency of *Dmrt1* leads to sex reversal from male to female while *Dmrt1* knock out (KO) mice exhibited severe deficiencies in testes differentiation [10]. Though it has been shown that in humans, *Dmrt1* and *Dmrt2* are confined to chromosome 9p24 [11], other transcription factors like SRY related box-9 (*Sox-9*), known to play a potential role in male-specific Sertoli cell development and female-specific Fork head box L2 (*Foxl2*) promoting female-specific granulosa and theca cell fates are located in autosomes. Interestingly, *Dmrt1* is known to control hundreds of downstream target genes in both Sertoli and germ cells differentiation in humans and also activate several testes-specific *Sox9* and *Sox8* genes but repressed ovary-specific genes including *Foxl2, R-Spondin-1* signalling proteins and oestrogen receptors [27] confirming their dual role in sex determination in humans. Similarly, in the present study, we show that *Dmrt1* is confined to the Y chromosome in tetras, as it amplified a 237 and 300 bp amplicons only in males as in *O. latipes* [7] confirming its potential as the male-sex-specific molecular marker (Figure 1). Transcriptome profiling of gonads using next generation sequencing (NGS) technology may reveal novel biomarkers and may uncover several genes that are overexpressed or dysregulated during gonadal development in fishes and humans. Despite endless possibilities, *Dmrt1* offers a common and canonical framework for sex determination, differentiation and development of male gonads and sex-specific traits in humans [11]. It is known that in humans, maternal blood circulation exchange nutrients and immune factors between the mother and the foetus, which can be used as source for isolating genomic DNA of foetus using

established protocols [22], as maternal circulation harbours blood cells of the foetus. Therefore, *Dmrt1* gene can be used as an alternate and inexpensive, male-sex determining molecular marker in humans thus avoiding invasive procedures. Hence, *Dmrt1* may be successfully utilized in both teleosts and advanced vertebrates including humans for identification of males thus providing an inexpensive alternative to the current laborious and expensive biomedical procedures.

CONCLUSION

From our present study, we conclude that *Dmrt1* can act as a potential sex-specific and Y chromosome-specific molecular marker in tetras by a rapid PCR technique with possibilities of application for non-invasive identification of male sex in several vertebrate and mammalian species including humans for determining male sex by an inexpensive PCR assay.

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