Procedure for the identification of sexual differentiation genes in *Hemichromis guttatus* females

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Abstract

The identification of sexual DNA markers has served many purposes, from basic knowledge on sexual development and differentiation, to the assessment of sex reversion of hormonal exposed fish. In the present study several candidate genes were analyzed to be validated as sexual markers for the cichlid *Hemichromis guttatus* a well-known invasive species whose genome or any genetic information is not yet known and that has important ecological impacts in a natural protected area characterized by several endemisms. A way to eradicate invasive species is the Trojan Y-Chromosome hormonal strategy in which males with two Y chromosomes are breed with females of the invasive population to lead to the decline or disappearance of the invasive population. The identification of candidate marker genes in the cichlid genome was confirmed by PCR essays using genomic DNA, and the expression of these same genes using mRNA extractions. Genomic material was extracted from gonadal tissue from *H. guttatus* specimens, regardless of their sex, size or age. Four candidate genes have been selected for sexual identification of phenotypically differentiated females, and thus primers for the orthologues genes: *Foxl2a*, *Figla*, *Wnt4a* and *Ctnnb1b* have been obtained. All genes have been successfully amplified using both nucleic acids.

Keywords: *Foxl2a* • *Figla* • *Ctnnb1b* • *sexual identification* • *Cichlidae*.

Introduction

Hemichromis guttatus, commonly known as jewel cichlid, is an ornamental fish native to Africa that has been introduced to different regions of North America, including the Natural Protected Area Cuatrociénegas of Coahuila, Mexico, and Austria. At the present it represents an evident threat to the local biodiversity, as a result of its high reproduction rate, its aggressive behavior and its ability to outcompete endemic naïve native species that constitute almost the totality of its diet. Consequently, native populations have drastically declined Until now, efforts to continuously fish the invasive populations have been unsuccessful (Área de Protección de Flora y Fauna Cuatrociénegas, 2008), mostly as a result of their high reproduction rate and larvae survival. Considering this context, the development of strategies leading more effective and long-lasting results is paramount. One of these solutions relays in the Trojan Y-Chromosome strategy which consists in exposing invasive males to female sexual hormones so as to obtain males with two Y chromosomes (YY), which after breeding with females of the invasive population will result in a male-dominant population that will eventually lead to the decline or disappearance of

the invasive population. A crucial step in this strategy is the identification of genetic sexual markers for the early and precise identification of reverted males as well as the offspring that carry the YY chromosomes. Identification of sexual markers poses a particular challenge as they are sex specific and they vary among species. In the present study genes involved specifically in female gonadal development in the cichlid *Hemichromis guttatus* were analyzed in order to identify sexual markers for this species.

Material and Methods

Wild invasive jewel cichlid was caught and transported to the Ecophysiology laboratory, FCB, UANL. A total of 10 adult males and 10 females were selected for gonadal tissue extraction. Fish size ranged from 3 to 5cm. For genomic DNA (gDNA) extraction, 100mg of muscle were dissected and placed in 15ml Falcon tubes containing of 70% ethanol to cover the whole tissue sample. Thereafter a traditional phenol:chloroform extraction was performed (Chen, et al., 2007). Because of the high content of lipids in muscle samples several extractions were needed. Gonad samples ranging from 20 to 50mg were placed in 1.5mL Eppendorf tubes containing RNAlater solution (enough to cover the whole tissue), for the subsequent extraction of total RNA using either the trizol method (Sambrook and Russell, 2006) or columns of the RNeasy® Kit by Qiagen. For the preparation of cDNA from the RNA extraction the Accuscript (Qiagen) protocol was used employing reagents from the Omniscript Kit (Qiagen). Finally, DNA, total RNA and cDNA were stored at -20 °C until further use. The resulting genomic material was used in PCR reactions that were prepared inside a Telstar Bio II Advance biosecurity cabinet, and then were run in either an Axygen Maxygen Thermocycler from Axygen Biosciences or a T100 Thermal Cycler (BIORAD) for the amplification essays. The standard PCR protocol consisted of 3 min at 95 °C, followed by 35 cycles under the following conditions:Denaturation: 95 °C for 30 seconds; Annealing: optimum temperature for each pair of primers for 30 seconds; Extension: 72 °C for 30 seconds. Finally, after the 35 cycles a final extension of 72 °C for 1 minute was performed. Results were visualized using a Gel Doc EZ Imager (BIO-RAD). Oligonucleotides used for the PCR essays of each candidate gene were taken from Böhne *et al*., (2013). And their synthesis was requested to T4OLIGO-Novik. Once the genes were amplified purification protocols directly from PCR reactions followed, using a Qiaquick Kit from Qiagen, while for products treated from agarose gels a QiaEX II Kit was used. Purified products were used for the cloning essays using different protocols: preparation of calcium competent cells (DH5-α), ligation, transformation and preparation of LB medium with ampicillin IPTG and X-Gal, from Cloning plus kit (Qiagen). And from this clones a Sanger automatic sequencing protocol is nowadays being performed for obtaining the genomic sequences of some candidate genes.

Results and Discussion

PCR protocols were carried out for the amplification of orthologue genes, *Ctnnb1b*, *Figla*, *Wnt4a* and *Foxl2a*, in gDNA and cDNA, aimed at verifying the presence of the markers in females of H. guttatus. For the standard PCR protocols, performed with gDNA and cDNA, the temperature gradient selected for the annealing step was based on the melting temperature for each primer pair, obtained by the formula (A+T) 2 + (C+G) 4 = Tm – 5, and by oligo analyzer from IDT.

All fragments could be amplified in both templates and ranged within the molecular weight estimated by *in silico* essays with the orthologue gene sequences of all genes, in this way the best temperature to amplify only one product for every gene was selected. PCR protocols started with the gDNA template to find out if the genes were present in *H. guttatus* genome, and once the amplification in gDNA was achieved cDNA was used for exploring gene functionality or gene expression purposes. Fig. 1 shows the amplification product of *Wnt4a* as an example of a positive PCR assay.

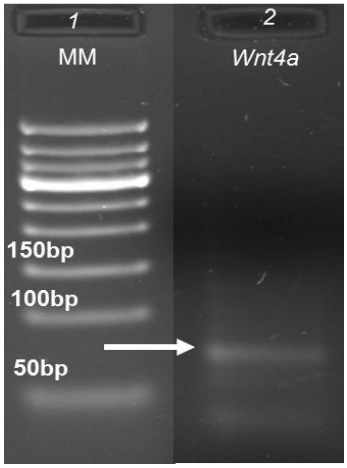

Figure 1. 2% Agarose gel stained with ethidium bromide showing standard PCR protocol results from reactions for the *Wnt4a* gene. Lane 1 (MM) corresponds to a 50bp molecular marker. Lane 2 shows the *Wnt4a* amplified product with an estimated size of 75bp, using cDNA as template.

Once the amplification of the products succeeded, purification from PCR reactions followed, as above mentioned, and the results are shown below (Fig. 2).

Purified PCR products

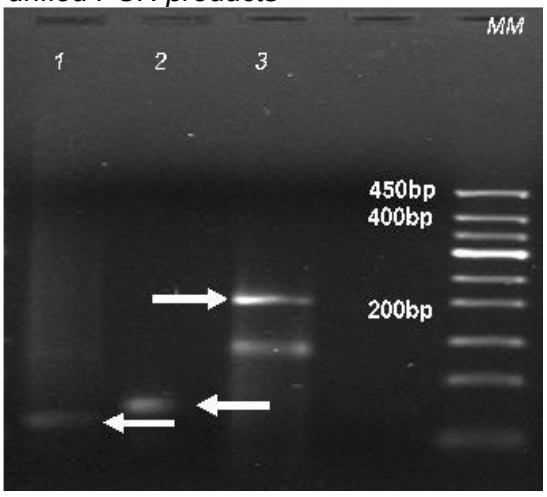

Figure 2. 2% Agarose gel stained with ethidium bromide showing purification results from PCR reactions of *Foxl2a*, *Figla* and *Ctnnb1b* genes. Lane 1 shows the *Foxl2a* amplified product with an estimated size of 59bp. Lane 2 shows the *Flgla* amplified product with an estimated size of 70bp. Lane 3 shows the *Ctnnb1b* primers with an estimated size of 200bp. An extra fragment near 150pb is observed as a result of a possible degradation of the gene since PCR assays usually demonstrate only one amplified product. Lane 4 (MM) corresponds to a 50bp molecular marker.

Once the products were purified cloning assays were initiated for sequencing. To date, only the results from the *Ctnnb1b* gene are available, and as stated in the methods section a cloning protocol where *E. coli* DH5α were transformed with the purified gene ligated to

pDrive plasmid was used. Several cell cultures were performed, and those colonies thought to be successfully transformed according to the cloning kit instructions were selected, until the transformation was fully achieved. Once this was accomplished a standard plasmid purification was done. Two PCR protocols were performed using the purified plasmid as template, one with *Ctnnb1b* primer pairs and the other with plasmid primers. PCR assays were done to verify if the plasmid contained the fragment of interest. Using *Ctnnb1b* primers it coul be observed that the gene was correctly ligated in the plasmid agreeing with the molecular weight of the product previously estimated in other PCR assays, and with the plasmid primers. Taken into account the size of the gene plus the size of the cloning site of the plasmid, it could be confirmed that the fragment was ligated to the plasmid. Both PCR results helped to identify the gene of interest as a function of its estimated molecular weight and to assess that it was ligated to the plasmid by comparing and adding the molecular weight of both plasmid cloning sites and the gene fragment.

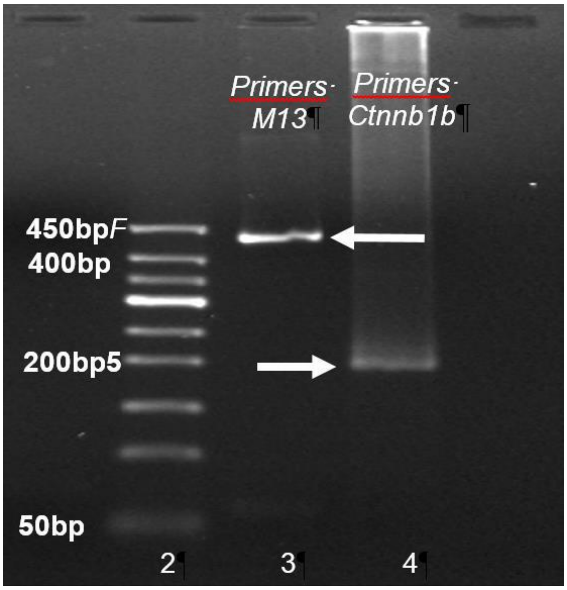

Figure 3. Agarose gel at a 0.8%concentration, stained with ethidium bromide. Lane 2 shows a molecular marker of 50bp. Lane 3 represents the PCR product obtained with the primers from pDrive plasmid showing a weight close to 438bp corresponding to the estimated weight resulting from adding the 238bp cloning site of the plasmid to the 200bp of the fragment to which it was ligated to, Lane 4 shows a PCR product obtained using *Ctnnb1b* primers where the product of the gene can be seen at 200bp.

In this way the results of PCR protocols using both gDNA and cDNA as templates were useful to confirm that the orthologue genes selected, *Ctnnb1b*, *Figla* and *Foxl2a*, were in fact present in the genome and expressed in *Hemichromis guttatus*. And even though the specific sequence of every gene is still unknown it seems very likely that the primer pairs may not vary. A possible critical observation to the procedure could be the amplification of only one PCR product for every analyzed gene considering that their expression will be measured using qPCR. This imply that if the primers anneal with more than one region on the template the quantifications would not be directly correlated with the product of interest. In the case of *Ctnnb1b,* there is a need to design specific primers once we the gene sequence is obtained.

Conclusions

All orthologue genes are functionally present, meaning that these are actually expressed in *H. guttatus*. The cloning and sequencing analysis of these genes in *H. guttatus* are pending in order to provide new genomic information for the eradication of this invasive species.

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