Bull Environ Contam Toxicol (2017) 98:41–45
DOI 10.1007/s00128-016-1994-1

Partial Gene Sequencing of CYP1A, Vitellogenin, and Metallothionein in Mosquitofish *Gambusia yucatana* and *Gambusia sexradiata*

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Received: 18 May 2016 / Accepted: 29 November 2016 / Published online: 2 December 2016

Abstract Ground characteristics in the Yucatan Peninsula make recovery and treatment of wastewater very expensive. This situation has contributed to an increase of pollutants in the aquifer. Unfortunately, studies related to the effects of those pollutants in native organisms are scarce. The aim of this work was to obtain partial sequences of widely known genes used as biomarkers of pollutant effect in *Gambusia yucatana* and *Gambusia sexradiata*. The studied genes were: cytochrome P450 1A (CYP1A); vitellogenin (VTG); metallothionein (MT), and two housekeeping genes, 18S and β-actin. From reported sequences of *Gambusia affinis*, primers were designed and amplification was done in the local *Gambusia* species exposed for 48 h to gasoline (100 µL/L, stirred for 24 h pre-exposure). Preliminary results revealed partial sequences of all genes with an approximate average length of 200 bp. BLAST analysis of found sequences indicated a minimum of 97% identity with reported sequences for *G. affinis* or *Gambusia holbrooki* showing great similarity.

Keywords *Gambusia* · Mosquitofish · CYP1A · Vitellogenin · Metallothioneins

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The Yucatan Peninsula, Mexico, is a flat and large emergent carbonate platform (Escolero et al. 2002; Perry et al. 2003) and due to its geological characteristics, surface-water runoff and drainage are practically non-existent. All of the water supply for human, agricultural, and industrial use is from a karst aquifer, one of the world's largest. There are only a very limited number of superficial water bodies, represented by "aguadas" that are small water bodies without connection to subterranean waters and "cenotes" that are sinkholes formed by dissolution and collapse of the carbonate rock. A semicircular ring of cenotes exists in northern Yucatan, with its center is near the site of the Chicxulub meteorite impact 65 million years ago (Bauer-Gottwein et al. 2011; Perez et al. 2011). Like the other karstic regions, the risk of groundwater pollution is high because the rock is highly permeable (Escolero et al. 2002; Perry et al. 2003; Bauer-Gottwein et al. 2011). Anthropogenic activities such as tourism, industry, agriculture and the rapid increase of population density are the principal risks to ecosystems, including the wildlife (Hernández-Terrones et al. 2011; Bauer-Gottwein et al. 2011; Gondwe et al. 2011; Pastén-Zapata et al. 2014; Leal-Bautista et al. 2013).

A biomarker is any biological response to an environmental chemical at the subindividual level, measured inside an organism, indicating a deviation from the normal status (Van der Oost et al. 2003). Biomarkers may provide for effective monitoring of water quality and assessment of the bioavailability and toxic effects of particular pollutant groups (Langston et al. 2007). The molecular biomarkers are the first signal that can be detected when the presence of pollutants exists, because they are shifting from transcript response to transcriptional modification, such as methylation, epigenetics, and miRNA. The induction of the genes of cytochrome P450 1A (CYP1A), vitellogenin (VTG) and metallothionein (MT) are the most studied (Huang et al. 2014).

CYP1A is the best-studied biomarker for environmental contamination in aquatic ecosystems (Van der Oost et al. 2003; Uno et al. 2012; Huang et al. 2014). This gene is induced by aromatic and polychlorinated aromatic hydrocarbons (PAH) in a dose-dependent manner, resulting in the production of a xenobiotic metabolizing enzyme (Carney et al. 2004; Uno et al. 2012; Kim et al. 2013). Vitellogenins (VTG) are important yolk precursor proteins that are synthesized in the liver of sexually mature female oviparous fish and are crucial for optimal oocyte growth; this protein is undetectable in males or juveniles but can be induced in response to estrogenic compounds. The increase of VTG expression in juvenile male fish is used commonly as a biomarker to assess estrogenic chemicals and estrogen pollutants in the aquatic environment (Kristensen et al. 2007; Gräns et al. 2010; Huang et al. 2012, 2013; Brockmeier et al. 2013, 2014). Metallothioneins (MT) are low-molecular mass proteins that are induced by pollutants, especially by essential metals. These proteins play a principal role in the protection of cells against the toxic effects of metals. For that reason, MT has been applied as a biomarker of metal pollution in the aquatic environment (Mattos et al. 2010; Huang et al. 2013, 2014; Siscar et al. 2014).

The use of small-size fish as sentinel species for ecology is a trend, because they are generally easy to maintain and breed under laboratory conditions (Kong et al. 2008; Sholz and; Mayer 2008; Celander 2011), the most used fish are the zebrafish (*Danio rerio*) (McElroy et al. 2012, Hsu et al. 2013), guppy (*Poecilia* spp.) (Mattos et al. 2010; Wast et al. 2014), Japanese medaka (*Oryzias latipes*) (Zhu et al. 2013; Sun et al. 2014), mosquitofish (*Gambusia affinis*) (Zaidi and Soltani 2010; Kamata et al. 2011; Wen et al. 2013) and eastern mosquitofish (*Gambusia holbrooki*) (Brockmeier et al. 2013). Unfortunately, studies related to the effects of pollutants in wild fish from Yucatan are scarce (Osten et al. 2005). We selected *Gambusia yucatana* and *Gambusia sexradiata*, two regional species that are sympatrically widespread in small streams in the Yucatan Peninsula for their potential as regional bioindicators of water pollution (Pérez-León and Schmitter-Soto 2007).

The aim of this work was to obtain partial sequences of CYP1A, VTG A, VTG B, MT genes and two housekeeping genes 18S and β-actin in native fish *G. yucatana* and *G. sexradiata*. The partial sequences were obtained in fish that were exposed to gasoline-treated water.

Materials and Methods

Complementary DNA sequences from cytochrome P450 1A (CYP1A), vitellogenin A (VT A), vitellogenin B (VTGB), metallothionein (MT), β-actin and 18S of *G. affinis* were obtained using the public genetic sequence database at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA). Conserved sequence regions were identified and used for the design of primers with "OligoPerfect Designer" software (Life Technologies Corporation, Grand Island, NY, USA).

Adult female *G. yucatana* and *G. sexradiata* (approximately 28–35 mm total length, n = 10) were captured in cenotes from Yucatan, Mexico, and acclimated for 3 months in 40 L tanks with aerated recirculating water under controlled laboratory conditions. After acclimation, fish were exposed for 48 h to gasoline (100 µL/L, stirred for 24 h pre-exposure), with daily exposure water changes. Gasoline is a complex mixture of pollutants that contains PAH's, endocrine disruptors, and metals, among other contaminants.

At the end of the exposure, the mosquitofish were euthanized by hypothermic shock and the body was slit open along the mid ventral line (from the anal vent to the operculum) to facilitate the extraction of the all the contents of the abdominal cavity, and were preserved in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) at −20 °C until RNA was extracted.

Total RNA was extracted from abdominal tissues of the fish by using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Total RNA was quantified by measuring the spectrophotometric absorbance at 260 nm; the purity and the integrity of the RNA was assessed at 260/280 nm. All the samples were adjusted to 200 ng/µL. 5 µL of RNA were then reverse transcribed into cDNA in a final volume of 20 µL, using I-Script Reverse Transcription Supermix for the RT-qPCR kit (Bio-Rad, Hercules, CA,USA), as described by the manufacturer. The reaction mix was incubated in a thermocycler at 25 °C for 5 min for priming, 30 min at 42 °C for reverse transcription and a final step for RT inactivation at 85 °C for 5 min. The first strand of cDNA was stored at −80 °C until subsequent PCR.

Amplification of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes for RT-PCR in *Gambusia* spp. were performed in a thermocycler (MJ Mini BioRad, Hercules, CA, USA), using the following conditions: an initial denaturation step of 94 °C for 5 min; 35 cycles at 94 °C for 30 s of denaturation; 55 °C for 30 s, and 72 °C for 30 s, and a final step of 72 °C for 5 min. The PCR products were visualized using 1% agarose gel stained with SYBR green. PCR products showing a single band were excised and purified using a EZ-10 Spin column DNA gel Extraction kit (Bio Basic Inc., USA). PCR products that showed two bands were excised and purified. The purified PCR products were sequenced at the "Sequencing Laboratory" of

Table 1 List of primers used for the amplification of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes for RT-PCR in *Gambusia* spp.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Length (pb)
CYP1A	CCTCGCTGAAGATTTTGTCC	TCCGGTCCTCACAGTGATCT	186
VTG A	GTCGAAGCTTGTGGAACCTC	CACTTGTTCAGGTCCCTGGT	319
VTG B	TCTGGAGGCAATTCAAATCC	ACCAGAACCAGGGGTAGCTT	595
MT	GAAAAGCTGCTGCTCTTGCT	AGGCTCCTCACTGACAGCAG	65
β-actin	ACTGGGACGACATGGAGAAG	CGTACATGGCAGGAGTGTTG	125
18S	GTTAATTCCAGCTCCAATAGCGT	GAACTACGACGGTATCTGATCGTC	399

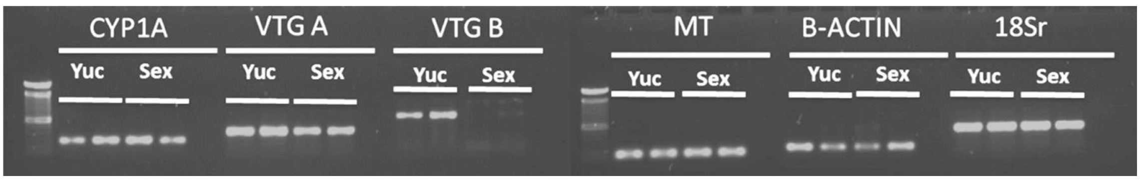

Fig. 1 Gel electrophoresis of the PCR products obtained with the primer pairs for the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes in *G. yucatana* (Yuc) and *G. sexradiata* (Sex). Molecular weight marker: 1 kb marker

Biotechnology Institute of UNAM, Mexico. The nucleotide sequences were compared to the corresponding references in the NCBI GenBank database using BLAST to verify that the correct cDNA fragments were isolated. The sequences of CYP1A, VTGA and MT among *G. yucatana, G. sexradiata* and *G. affinis* or *G. holbrooki* were aligned using Multalin Software (Corpet 1988).

Results and Discussion

Gene specific PCR primers CYP1A, VTGA, VTGB, MT, β-actin and 18S genes were all designed in accord with a good base composition, amplicon length, and melting temperature, and did not show a secondary structure. The gene-specific primers sets are depicted in Table 1.

PCR amplifications were conducted using pair primers that amplified the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes of *G. yucatana* and *G. sexradiata*. All primers produced an amplicon of different size, showing only one and defined expected band, except for VTGB in *G. sexradiata*, which showed two unspecific light bands (Fig. 1).

Sequences obtained of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes in *G. yucatana* and *G. sexradiata* were verified in the NCBI GenBank database, using BLAST, and they all corresponded to the first hit in sequences from *G. affinis* or *G. holbrooki*. The found identities ranged from 96.72% to 99.24% (Table 2). For β-actin and 18S, the identity between *G. yucatana* and *G. sexradiata* was 99.2% (data not shown) (Fig. 2).

There is a need to develop biological methods to monitor the toxic effects of pollutants in water bodies, and the use of selected sentinel species is crucial for the correct management of tropical ecosystems especially in emerging countries, especially where the study of basic aspects of the biology of fish is absent. Mosquitofish and eastern mosquitofish have been used for many years in ecotoxicology studies because they are sensitive to environmental changes, but very few studies have been done with mosquitofish in tropical ecosystems.

Water pollution alters reproductive behavior and the endocrine system of mosquitofish, affecting their populations (Saaristo et al. 2014; Frankel et al. 2016). It is important to measure the effects of pollutants in wild tropical mosquitofish, which are important in controlling the larvae of mosquitoes that may serve as vectors of diseases such as dengue, chikungunya and zika.

Table 2 Identity of *G. yucatana* and *G. sexradiata* of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes, respective of the first hit

Species	Gen	Hit	Identity (%)
G. yucatana	CYP1A	*G. affinis* (AB371607.1)	98.35
G. sexradiata	CYP1A	*G. affinis* (AB371607.1)	99.45
G. yucatana	VTGA	*G. affinis* (AB181835.1)	97.81
G. sexradiata	VTGA	*G. affinis* (AB181835.1)	98.05
G. yucatana	VTGB	*G. affinis* (AB181836.1)	98.82
G. sexradiata	VTGB	N/A	–
G. yucatana	MTF	*G. affinis* (AB455145.1)	96.92
G. sexradiata	MTF	*G. affinis* (AB455145.1)	98.46
G. yucatana	β-actin	*G. affinis* (AB371607.1)	99.2
G. sexradiata	β-actin	*G. affinis* (AB371607.1)	99.2
G. yucatana	18S	*G. holbrooki* (FJ710843.1)	99.24
G. sexradiata	18S	*G. holbrooki* (FJ710843.1)	99.24

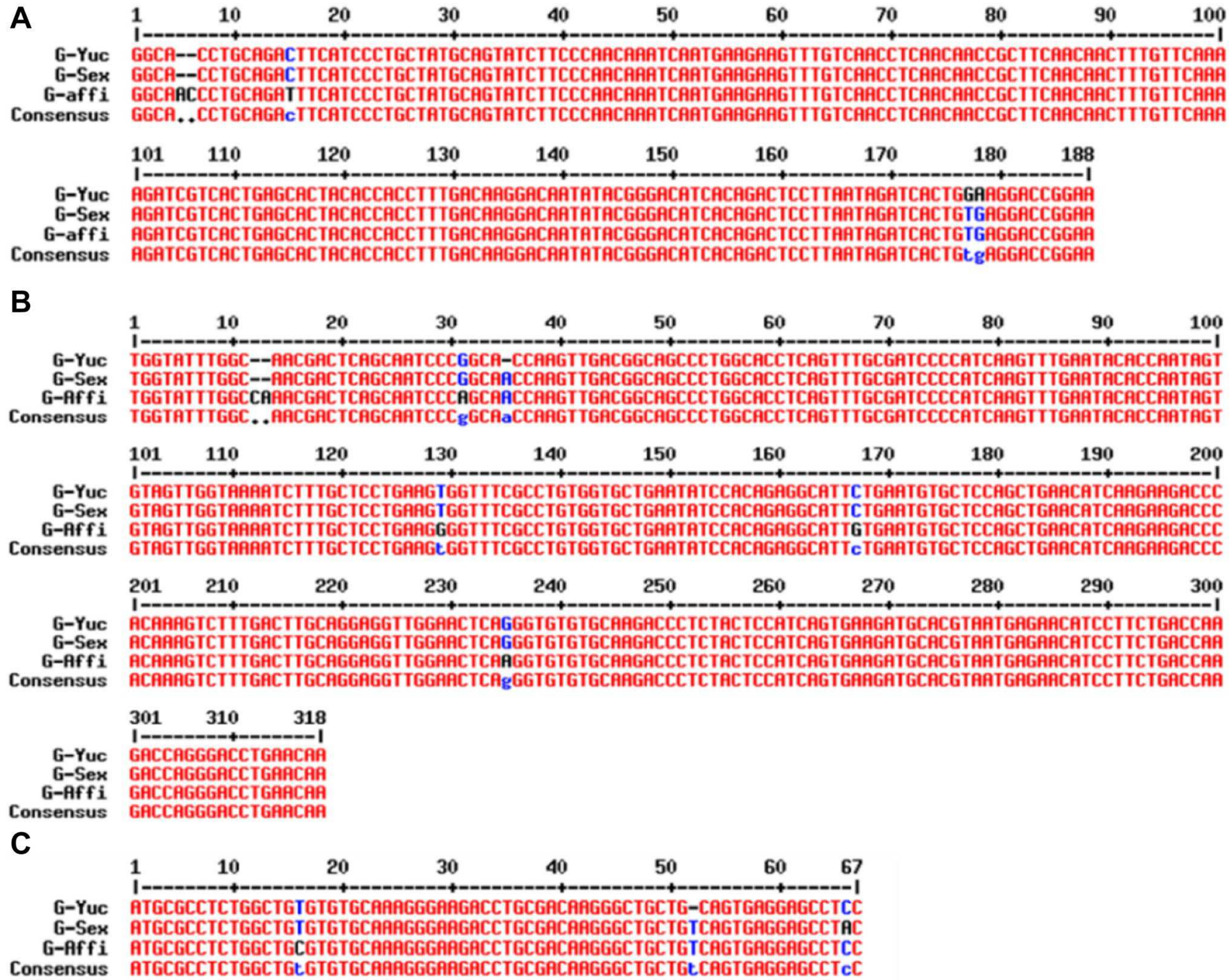

Fig. 2 Alignment among sequences of the CYP1A (**a**), VTGA (**b**) and MT (**c**) genes of *G. yucatana, G. sexradiata* and *G. affinis. Red letters* show conserved zones, *blue letters* indicate nucleobases that present 66% difference. (Color figure online)

In this study, we designed primers to amplify fragments of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes in *G. yucatana* and *G. sexradiata*, two native fish that can be used as bioindicators, and the sequences obtained confirmed close homology with respect to *G. affinis* and *G. holbrooki*, which are standard fish proposed as sentinels of water pollution (Kamata et al. 2011; Wen et al. 2013; Brockmeier et al. 2013). This is the first report of sequences of the CYP1A, VTGA, VTGB, MT, β-actin and 18S genes of the species *G. yucatana* and *G. sexradiata*. The sequences identified will be useful in obtaining full-length sequences and analysis of changes in the expression of CYP, VTG and MT genes in wild mosquitofish in the Yucatan Peninsula.

Acknowledgements The authors wish to acknowledge UNAM-PAPIIT-DGAPA grant IA200214 and the Postdoctoral Fellowship Program of the Mexican National Council of Science and Technology (CONACyT) for the financial support of this study. We also acknowledge Ismael Oceguera-Vargas, Anita Arroyo-Silva and Fernando Mex-Esquivel for their valuable efforts during the field work.

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