El Colegio de la Frontera Sur

Optimización de marcadores moleculares ISSR en el caracol rosado *Strombus gigas*

TESIS

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Las personas abajo firmantes, integrantes del jurado examinador de **Jorge Cruz Medina**, hacemos constar que hemos revisado y aprobado la tesis titulada: **Optimización de marcadores moleculares ISSR en el caracol rosado *Strombus gigas***, para obtener el grado de **Maestro en Ciencias en Recursos Naturales y Desarrollo Rural.**

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En memoria de Alberto Anzures Dadda (1968-2012)
y Ciriaco Medina García (1912-2013).

Dedicado a mis padres y hermanos; a Tania.

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CONTENIDO

Resumen

El caracol rosado *Strombus gigas* es un gasterópodo marino de importancia económica y ecológica en el Caribe. Durante las últimas décadas este molusco ha padecido una intensa presión por sobreexplotación, pérdida de hábitat y contaminación, provocando un serio declive en el recurso. A pesar de las diferentes regulaciones de pesca implementadas y de esfuerzos en investigación con fines de restauración, actualmente *S. gigas* es considerada a nivel mundial como especie comercialmente amenazada. El manejo y conservación del caracol rosado pueden ser mejorados a partir del conocimiento de aspectos genéticos de la especie. Una consideración importante para el desarrollo de estudios genéticos en moluscos es que los tejidos fuente de ADN contienen altas cantidades de componentes que afectan negativamente la amplificación de ADN. Los objetivos de este trabajo fueron identificar una vía adecuada para resolver los problemas de inhibición de la PCR y adaptar una herramienta molecular sencilla y de alta resolución en *S. gigas*, ambos enfocados al desarrollo de análisis genéticos de individuos y poblaciones. Para ello, se emplearon muestras de cuatro diferentes localidades del Caribe Mexicano y se evaluó el desempeño de: a) tres protocolos de extracción de ADN, b) dos estrategias adicionales para superar problemas de inhibición en la PCR, y c) 23 marcadores ISSR. Se identificó el empleo de un método de extracción por sales, con la adicional purificación de ADN mediante columnas de afinidad, como una ruta adecuada para asegurar la obtención de ADN funcional de caracol rosado. Se observó un patrón de inhibición de la PCR en función de la localidad de procedencia de las muestras; posibles explicaciones son propuestas. Se confirma la aplicabilidad de la técnica ISSR obteniendo 11 marcadores funcionales para

S. gigas, lo que representa una herramienta valiosa para estudios futuros que requieran información genética robusta y enfoques moleculares rentables.

Palabras clave: Caribe Mexicano, especie amenazada, extracción de ADN, gasterópodo marino, inhibición de la PCR.

Introducción

El caracol rosado *Strombus gigas* L. 1758 (Mesograstropoda, Strombidae), también referido actualmente como *Eustrombus gigas* (Cala et al., 2013), es una especie de importancia ecológica y económica en el Caribe. Sus hábitos alimenticios regulan la estructura y abundancia de diferentes organismos epibénticos (Stoner et al., 1995), y al mismo tiempo representa una fuente alimenticia para diversas especies de crustáceos, moluscos y peces, entre otros (Randall, 1964). Este gasterópodo es además un recurso de gran valor comercial –e incluso cultural- para diferentes naciones en el Caribe (Aldana y Brulé, 1994), considerado el segundo recurso pesquero más importante de la región (Brownell y Stevely, 1981). El principal atractivo económico del caracol rosado reside en la explotación de su carne, aunque su concha también ha sido aprovechada en la industria de la porcelana y con fines ornamentales (Randall, 1964).

Durante siglos este molusco ha sido explotado como alimento; no obstante, la pesquería comercial a gran escala detonó sólo en las últimas décadas, principalmente como respuesta al aumento de la demanda internacional (CITES Significant Trade Review, 2003). Desde entonces el caracol rosado ha padecido una intensa presión por sobreexplotación, lo que ha producido -junto con otros factores antropogénicos como pérdida de hábitat, contaminación ambiental y falta de cumplimiento de las regulaciones de pesca existentes- un declive en la mayoría de los stocks de *S. gigas* (Glazer y Quintero, 1998; Theile, 2001). Actualmente a nivel mundial, el caracol rosado es catalogado por la Convención sobre el Comercio Internacional de Especies Amenazadas de Fauna y Flora Silvestres (CITES) como especie comercialmente

amenazada. En el caso particular de México, se han implementado diferentes regulaciones desde 1976 con el fin de controlar la pesca de este molusco (Basurto et al., 2005). En la actualidad su extracción legal se encuentra prohibida en el país debido al estado de deterioro del recurso (DOF, 2012).

Diversas investigaciones han sido desarrolladas con el propósito de favorecer el restablecimiento de poblaciones naturales y mejorar el manejo de *S. gigas*. Estos estudios incluyen aspectos sobre su biología y/o ecología (Randall, 1964; Iversen et al., 1986; Carrascal et al., 2012), demografía (de Jesus-Navarrete et al., 2003; Stoner et al., 2012), maricultura (Berg, 1976), entre otros. A pesar de estos esfuerzos, sólo pocas áreas exhiben agregaciones que pueden ser consideradas estables o con signos de recuperación (Theile, 2001).

Una alternativa para la conservación de poblaciones naturales reside en el establecimiento de unidades de manejo, definidas a partir del conocimiento de la variabilidad genética de la especie y/o poblaciones de interés (Frankham et al., 2010). Para el caso de *S. gigas*, se han conducido estudios genéticos utilizando diferentes herramientas moleculares y analizando la variabilidad genética sobre distintas áreas geográficas: aloenzimas, empleadas para estudios alrededor del Caribe y Bermuda (Mitton et al., 1989), los Cayos de Florida e Islas Bimini (Bahamas) (Campton et al., 1992), y el Caribe Mexicano (Tello-Cetina et al., 2005); mientras que genes de ADN mitocondrial (ADNmt; Pérez-Enriquez et al., 2011) y microsatélites (Zamora-Bustillos et al., 2011) fueron utilizados para el análisis de muestras colectadas en las proximidades de la Península de Yucatán. De manera general, sus resultados señalan que el

conjunto de poblaciones geográficas analizadas en cada estudio son similares genéticamente. No obstante, existe discrepancia sobre si *S. gigas* constituye una población panmíctica, lo que obstaculiza el adecuado manejo del recurso.

Es importante precisar que las características inherentes a cada herramienta molecular pueden conferir ventajas y limitaciones a la investigación. Por ejemplo, análisis con aloenzimas/isoenzimas son de fácil implementación y el procesamiento de muestras es de relativo bajo costo; sin embargo, la información producida no siempre presenta el nivel de resolución de herramientas moleculares más recientes, ya que esta técnica analiza indirectamente la variabilidad genética (Parker et al., 1998; Pronob et al., 2010). Por su parte, diversos genes en el ADNmt pueden exhibir un gran número de alelos por locus, lo que permite la obtención de información con mayor detalle, pero las características propias del ADNmt (e.g. herencia maternal) hacen que ésta molécula sea atractiva para el estudio de procesos poblacionales históricos, más que contemporáneos (Moritz, 1995; Wink, 2006; Casu et al., 2011). Análisis genéticos con microsatélites proveen información con alto nivel de resolución, aunque esta herramienta demanda un mayor esfuerzo y costo al inicio de su implementación (Wink, 2006).

Las Secuencias Repetidas Inter-Simples o ISSR, son una herramienta molecular con elevado potencial para el estudio de genética de poblaciones (Culley y Wolfe, 2001; Behura, 2006). Esta técnica involucra la amplificación simultánea de múltiples regiones de ADN mediante la Reacción en Cadena de la Polimerasa (PCR). Tales regiones se identifican mediante el uso de marcadores o *primers* (secuencias simples repetidas,

como $(CA)_n$ o $(AG)_n$) inversamente orientados dentro del genoma nuclear eucarionte (ver anexo 1). Entre las ventajas de esta técnica se encuentran 1) su aplicabilidad para un amplio número de especies, sin necesidad del conocimiento previo sobre secuencias de ADN, 2) el aporte de información con elevado nivel resolutivo, ya que detecta gran cantidad de variabilidad genética, 3) la alta reproducibilidad de los resultados y 4) la facilidad de su implementación a un relativo bajo costo (Gupta et al., 1994; Zietkiewicz et al., 1994; Wolfe, 2005).

La principal limitación de los ISSR es que no permiten la distinción de genotipos homocigotos dominantes de heterocigotos, y que las estimaciones de diversidad genética se realizan a partir de caracteres dialélicos (presencia o ausencia de banda; Culley y Wolfe, 2001). A pesar de ello, esta herramienta ha sido empleada en diversos estudios para la asistencia del manejo y/o conservación de especies marinas: *Patella ferruginea* (Gastropoda; Casu et al., 2006), *Mactra veneriformis* (Bivalvia; Hou et al., 2006), *Corallium rubrum* (Anthozoa; Casu et al., 2008), *Crocodylus acutus* (Reptilia; Machkour-M'Rabet et al., 2009), *Paracentrotus lividus* (Echinoidea; Coupé et al., 2011), *Aristaeomorpha foliacea* (Malacostraca; Fernández et al., 2011). Considerando lo anterior, es fácil identificar el particular atractivo que los ISSR exhiben para el estudio de aspectos genéticos en *S. gigas*. Asimismo, aun cuando ya se hayan realizado trabajos similares en el caracol rosado, el uso de diferentes herramientas moleculares siempre es justificable cuando se requiere un mejor conocimiento de la estructura genética en una especie (Ward, 2000).

Un proceso fundamental para el desarrollo de estudios genéticos en moluscos es la extracción de ADN, ya que continuamente presenta dificultades (Winnepenninckx et al., 1993). Además es importante tener en cuenta que, pese a su uso frecuente para la amplificación de ácidos nucleicos, la PCR es susceptible de inhibición (Schrader et al., 2012). Este efecto negativo es causado por diversas sustancias (presentes naturalmente en la muestra analizada o incorporadas involuntariamente durante su manipulación), las cuales exhiben diferentes propiedades y mecanismos de acción inhibitoria (Wilson, 1997; Schrader et al., 2012). En el caso particular de moluscos, estudios señalan que los tejidos y secreciones de estos organismos contienen altas cantidades de polisacáridos y proteínas, los cuales usualmente son aislados junto con el ADN e interfieren la amplificación de secuencias mediante la inhibición de la actividad de la polimerasa (Winnepenninckx et al., 1993; Sokolov, 2000). Como respuesta a esta problemática, un número cada vez mayor de protocolos específicos han sido propuestos para la extracción de ADN en moluscos (e.g. Winnepenninckx et al., 1993; Sokolov, 2000; Pereira et al., 2011), mismos que difieren en eficiencia, costo, tiempo de procesamiento y grado de inocuidad para el usuario/ambiente.

Bajo este contexto, el presente trabajo fue conducido con el fin de proporcionar nuevas herramientas para el estudio genético poblacional en el caracol rosado *Strombus gigas*, por lo que 1) se identificó una vía adecuada para la resolución de problemas de inhibición de la PCR, y 2) se determinaron y estandarizaron marcadores moleculares ISSR para esta especie amenazada.

Las muestras biológicas empleadas en este estudio fueron colectadas durante el transcurso del año 2013 en cuatro localidades del Caribe Mexicano (anexo 2). Las colectas en el Parque Nacional Arrecifes de Xcalak (PNAX) y la Reserva de la Biósfera Banco Chinchorro (RBBC) fueron realizadas bajo la licencia PPF/DGOPA-064/13 (anexo 3). El material biológico procedente del Parque Nacional Arrecifes de Puerto Morelos (PNAP) y del Área de Protección de Flora y Fauna Isla de Cozumel (APFFC) fue obtenido a partir de decomisos de capturas ilegales, con el consentimiento de las autoridades pertinentes.

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Sincerely yours,

Dr. Salima Machkour-M'Rabet

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PCR-inhibitors and adaptation of ISSR molecular markers in queen conch *Strombus gigas*: tools for the study of population genetics

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ABSTRACT

The queen conch *Strombus gigas* is a marine gastropod of ecological and economic importance in the Caribbean. During the last decades, this mollusc has suffered the combined pressures of overexploitation, habitat loss and pollution, leading to a serious decline in this organism. Despite the diverse fishery regulations put into place and research efforts focused on species restoration, *S. gigas* is still catalogued as commercially threatened by CITES. The management of a resource can be improved through the knowledge of its genetic aspects. An important consideration for DNA extraction in molluscs is that source tissues contain high quantities of compounds which interfere in PCR. The aim of the present study was to find a suitable method to solve the problem of PCR-inhibitors and adapt a simple and high-resolution molecular technique for *S. gigas*, both dedicated to performing genetic analysis of individuals and populations. Samples were collected, by a non-lethal technique, at four different localities at the Mexican Caribbean and the performance of a) three DNA extraction protocols, b) two additional strategies to overcome PCR-inhibition problems, and c) 23 ISSR primers was evaluated. The employment of a salt extraction method with the additional purification by the bind-resin of Wizard® DNA Clean-Up System (Promega) is suggested as an appropriate route to assure the extraction of PCR-able queen conch DNA. A strong relationship between PCR-inhibition and locality of origin of samples was identified and a possible explanation is proposed. We also confirm the applicability of the ISSR technique by obtaining eleven functional primers for the queen conch, representing a valuable tool for future surveys where robust genetic information and cost-effective approaches are required.

INTRODUCTION

The queen conch *Strombus gigas* L. 1758 (Mesograstropoda, Strombidae), currently also referred as *Eustrombus gigas* (Cala *et al.*, 2013), is one of the largest and most commercially important marine gastropods in the Caribbean (Aldana-Aranda, 2003). This species has an essential ecological function, attributable to its feeding habits that regulate the structure and abundance of different benthic organisms (Stoner, Ray & Waite, 1995), and at the same time represents a food source for several marine species (Iversen, Jory & Bannerot, 1986). Some biological and behavioral characteristics of *S. gigas* (e.g. slow movement, easy identification, aggregations in shallow waters) have allowed exploitation by humans during many years. However, as a result of the increased pressure of overfishing, habitat loss and pollution, as well as a lack of enforcement of existing regulations, most aggregations of this mollusk present a population decline (Glazer & Quintero, 1998; Theile, 2001).

Since the 1970s, diverse researches have been developed in order to contribute to the restoration of natural populations, and to improve the management of *S. gigas* (Morales & Lopez, 2003). These studies include topics on its biology and/or ecology (Carrascal *et al.*, 2012; Iversen*, et al.*, 1986; Randall, 1964), demography (de Jesus-Navarrete, Medina-Quej & Oliva-Rivera, 2003; Stoner, Davis & Booker, 2012), mariculture (Berg, 1976; Iversen & Jory, 1997), among others. Despite these efforts, only a few areas exhibit aggregations that can be considered stable or with signs of recovery (Theile, 2001).

One alternative for conserving natural populations is the definition of management units through the knowledge of genetic variability (Frankham, Ballou & Briscoe, 2010). This issue has been studied in queen conch aggregations using different molecular approaches and different geographical areas: allozymes were used for studies in the Caribbean and Bermuda (Mitton, Berg & Orr, 1989), as well as in the Florida Keys and Bimini Islands (Bahamas) (Campton *et al.*, 1992); while mitochondrial DNA genes (Pérez-Enriquez *et al.*, 2011) and nuclear microsatellites (Zamora-Bustillos *et al.*, 2011) were employed for studies close to the Yucatan Peninsula (Mexico). A consensus reached by these investigations is that the whole analyzed localities in each study are genetically similar. Nonetheless, there is discrepancy about whether *S. gigas* constitutes a panmictic population and consequently the appropriate management of the resource is hindered.

A relative novel molecular technique, Inter Simple Sequence Repeats (ISSR), has shown wide applicability for the study of genetic aspects at intrageneric and intraspecific levels (e.g. Liu & Wendel, 2001). ISSR involves PCR amplification of genomic DNA without prior knowledge of sequences, because primer sequences are broadly distributed on the genome of many eukaryotic organisms (Gupta *et al.*, 1994). Additionally, the method provides abundant polymorphisms and highly reproducible results, which together reflects the simplicity, high resolution and reliability of this technique (Bornet & Branchard, 2001; Wolfe, 2005; Zietkiewicz, Rafalski & Labuda, 1994). Limitations of this molecular approach are that bands are scored as dominant markers and that genetic diversity estimates are based on diallelic characters (band presence or absence; Culley and Wolfe, 2001). Nevertheless, ISSR has already been used in a broad spectrum of applications: assessment of genetic variability (Fernández *et al.*, 2011; Liu & Wendel, 2001), analysis of the genetic structure of populations and estimation of genetic flow (Casu *et al.*, 2005; Hou *et al.*, 2006), genetic monitoring of populations (Ramp, Collinge & Ranker, 2006), identification of hybrids and introgression (Machkour-M'Rabet *et al.*, 2009) and taxonomic distinctions (Maltagliati *et al.*, 2005), among others.

ISSR markers have also been successfully employed for the study of diverse gastropods (Casu *et al.*, 2006; de Aranzamendi *et al.*, 2008; Dong *et al.*, 2011), and despite their advantages over other molecular approaches (in terms of cost, informativeness, ease of use and/or reproducibility; Yang *et al.*, 1996; Esselman *et al.*, 1999), this technique has not yet been applied in queen conch research. In addition, although population genetic studies have already been developed for *S. gigas*, the use of different type of markers for a better understanding of genetic structure issues is often justifiable (Ward, 2000).

A principal consideration for genetic studies in mollusks is that DNA extraction is often problematic (Winnepenninckx, Backeljau & Dewachter, 1993). Furthermore, although nowadays PCR is a widely used technique, it is important to consider that this process is susceptible to reaction inhibition (Schrader *et al.*, 2012). This drawback is caused by diverse substances (possibly present in the analyzed sample itself or added unintentionally during processing), which have different properties and mechanisms of action (Schrader*, et al.*, 2012; Wilson, 1997). In the case of mollusks, secretions and tissues contain high quantities of polysaccharides and proteins which usually co-purify with DNA, inhibiting the activity of diverse enzymes such as polymerases (Sokolov, 2000; Winnepenninckx*, et al.*, 1993) and consequently, amplification during PCR is negatively affected. To overcome this problem, different protocols of DNA extraction have been proposed (e.g. Winnepenninckx *et al.*, 1993; Sokolov, 2000; Pereira *et al.*, 2011), which differ in efficiency, cost, processing time and safety for the user/environment.

Our work was focused on two important aspects for the genetic study of *S. gigas*: I) to find a suitable DNA extraction protocol and an appropriate method to solve the problem of PCR-inhibitors, and II) to determine and standardize ISSR markers, in order to provide a new tool for the study of population genetics of this threatened gastropod.

METHODS

Sample collection

Samples of *S. gigas* were collected during 2013 at two localities on the Mexican Caribbean: Xcalak National Reef Park (XNRP; 18°21'05''N/87°48'11''W) and Banco Chinchorro Biosphere Reserve (BCBR; 18°24'N/87°22'02''W) under the license of permit PPF/DGOPA-064/13. In addition, samples from queen conch seized of illegal catch in the Cozumel Protected Area of Flora and Fauna (CPAFF; 20°33'44''N/86°49'28''W) and Puerto Morelos National Reef Park (PMNRP; 20°59'17''N/86°48'02''W) both in Quintana Roo (Mexico), were provided by the relevant authority. Due the threatened state of *S. gigas*, tissue samples were collected using a non-lethal technique (Harris, 2008), which consists of removing the conch from the top of the shell and keeping it out of the water in a dorsal-ventral position (in the same way the conch lies on the seabed). This position induced foot projection which permitted obtain approximately 1 g of foot muscle by a simple excision. Samples were washed with filtered seawater, preserved in ethanol 96%, labeled and maintained at 4°C until further analysis.

DNA extraction

Previous to DNA extraction, around 3 mm^3 of foot muscle (~20 mg) was briefly washed with ethanol and placed in 600 µl of ultrapure water during 1 hour for rehydration. Subsequently, to facilitate its posterior digestion, the piece of tissue was manually disrupted following the method of Sokolov (2000), and finally tissue was placed in a 1.5 ml Eppendorf tube for DNA extraction. Three DNA extraction protocols were evaluated: 1) phenol/chloroform/isoamylic alcohol (PCI) method described by Sokolov (2000), 2) salt extraction referred to by Aljanabi & Martinez (1997), and 3) a commercial kit (Wizard® Genomic DNA Purification, Promega) following the instructions for animal tissue. With the exception of the pre-treatment phase (sample preparation), all protocols were executed without any further modification. The concentration of obtained DNA was determined using a Qubit® 2.0 Fluorometer (Invitrogen) and the quality was visually checked using electrophoresis in agarose (1.5%)/TBE (1 X) gel. The extracted DNA was stored at -20°C until amplification.

ISSR-PCR amplification

Considering reported values in the literature, different tests were performed to adjust PCR conditions for ISSR amplification. Primer annealing temperature *Ta* was modified to allow oligonucleotide hybridization (to produce intense and reproducible bands) but minimizing production of faint fragments. The evaluated temperatures ranged from 48°C to 67°C, varying the temperature interval for each primer according to differences in melting temperatures *Tm* (Table 1), which were calculated by means of the OligoAnalizer 3.1 (Integrated DNA Technologies®). Another important parameter is the amount of template DNA used for the amplification reaction. Tests were carried out using between 6 to approximately 200 ng of template DNA per reaction, assessing the quality of the electrophoretic resolution. Concentrations of $MgCl_2$ (from 2 to 4 mM), primer (from 0.5 to 2 µM), dNTPs (from 200 to 400 µM each dNTP) and Taq polymerase (from 1 to 2 U) were systematically tested, in order to improve amplification and to reduce reagents consumption. Finally, once the PCR conditions were selected, the two different gel staining protocols with GelRed™, (precasting and post gel staining) were also evaluated.

All experiments were carried out using a T100™ Thermal Cycler (Bio-Rad) programmed as follows: an initial denaturation step at 94°C for 4 min, followed by 39 cycles of denaturation at 94°C for 45 s, *Ta* from 56°C to 66°C depending on ISSR primer (see Table

177 1), and extension at 72°C for 90 s; finally a final extension at 72°C for 10 min. Amplification
178 products were separated by electrophoresis (run at 110 V for 3h) using 1.5% agarose gels
179 in 1 X TBE Buffer (Promega) and a 100bp DNA Ladder (Promega) as reference for
180 fragment length. Final products were visualized and digitized using an imaging system
181 (AlphaImager® Mini).
182
183 *Detection and removal of PCR-inhibitors*
184 The presence of PCR-inhibitors in the extracted *S. gigas* DNA was suggested when a few
185 samples displayed products after DNA amplification under optimal PCR conditions (see
186 results), but the rest of samples exhibited neither bands nor smears. To verify this
187 presence, amplifications were performed using BDB$(ACA)_5$ primer together with one of
188 three kinds of template DNA: butterfly DNA (previously known as amplifiable; control
189 reaction), queen conch DNA (sample suspected to have PCR-inhibitors), and a blend of
190 DNA (the amplifiable butterfly DNA plus suspected queen conch DNA). To overcome the
191 presence of PCR-inhibition agents in the majority of *S. gigas* DNA extracts, three distinct
192 strategies were established and assessed in terms of efficiency in allowing amplification:
193 1) usage of three different DNA extraction protocols (previously referred), 2) employment
194 of DNA extracts in dilutions of 1:5, 1:15 and 1:30 (Monteiro *et al.*, 1997), and 3) usage of a
195 column-purification system (Wizard® DNA Clean-Up System, Promega), which was
196 chosen because it seems to be one of the most widely used kits available to purify DNA
197 (Harnpicharnchai *et al.*, 2007).

< Table 1

198 RESULTS
199
200 *DNA extraction*
201 The three tested protocols produced DNA of high molecular weight (HMW; Fig. 1).
202 Fluorometry analysis showed that the quantity of extracted DNA per sample was different
203 for each DNA extraction method, obtaining the following concentrations (mean ± SE): 89.7
204 ± 40.5 ng/µl in PCI extractions (*n* = 4), 33.1 ± 6.9 ng/µl using salt extraction procedure (*n* =
205 11), and 58.9 ± 10.9 ng/µl with Promega extraction Kit (*n* = 11). In terms of yield (ng of
206 obtained DNA per mg of tissue wet weight), mean values and their respective standard
207 errors were as follow: 448.4 ± 202.7 ng/mg for PCI (*n* = 4), 496.8 ± 98.5 ng/mg for salt
208 extraction (*n* = 11), and 294.5 ± 52 ng/mg for Promega extraction Kit (*n* = 11). There was
209 no evidence of RNA in the extracts (including those obtained with the salt extraction
210 method where no RNAse is employed), which would be observed as a smear of low
211 molecular weight material on the agarose gel (Mikhailova & Johannesson, 1998).
212 < Figure 1
213 A cost analysis of the evaluated protocols revealed that the salt extraction method was the
214 least expensive at US $0.44 per sample, followed by the PCI method at US $1.03 and the
215 Promega extraction Kit at US $2.45. In terms of difficulty, the number of steps involved in
216 the salt extraction protocol is nine, followed by Promega extraction Kit with 11 and PCI
217 method with 14. However, the salt extraction and PCI methods require extra preparation of
218 solutions and buffers, which makes the Promega extraction Kit a less laborious process.
219
220 *Adaptation of ISSR technique for Strombus gigas*
221 A total of 23 ISSR primers were tested (Table 1). Optimal PCR results were obtained in a
222 15 µl reaction volume containing: ~16 ng of template DNA (2 µl), 0.5 X Buffer (Green
223 Buffer, Promega), 200 µM of each dNTP from dNTP mix (Promega), 3 mM $MgCl_2$
224 (Promega), 1 µM of primer (Integrated DNA Technologies®), 1.25 U of Taq Polymerase
225 (GoTaq® Flexi, Promega), adjusting the final volume with ultrapure water.
226
227 Despite the different arrangements for PCR optimization, suitable amplification was unable
228 for 12 of the tested ISSR primers. The remaining 11 primers gave clear and reproducible
229 fragments with lengths ranging from approximately 100 bp to 2000 bp, and the number of
230 bands generated per marker ranged from 4 to 28 (Table 1).
231
232 Optimum *Ta* of tested primers positively affects the intensity, reproducibility and sharpness
233 of bands. Considering only functional primers (Table 1), better results were obtained when
234 *Ta* was used ~1.5°C below the calculated *Tm*, while using higher temperatures (≥ 2°C
235 above *Tm*) some bands disappeared as a result of high stringency. At lower temperatures
236 (≥ 4°C below *Tm*) band intensity and sharpness decreased; additionally, seldom new
237 amplified *loci* were registered, which can be attributed to anchoring in ISSR primers.
238
239 Regarding the amount of template DNA, it was found that concentrations higher than 25
240 ng per 15 µl of reaction occasionally inhibit PCR amplification. The best results were
241 obtained when DNA was added in quantities of between 6 and 25 ng in a reaction of 15 µl
242 (Table 2). Results for $MgCl_2$ and primer concentrations tests showed that lesser the
243 quantity of these elements in the reaction, lesser the intensity and number of amplified
244 products. At higher concentrations the opposite occurred, with an additional intense smear
245 on the gel. Intermediate concentrations of $MgCl_2$ (3 mM) and primer (1 µM) exhibited better
246 amplification (Table 2). In contrast, no evident modifications were observed when dNTPs
247 and Taq polymerase concentrations varied in the PCR. Thus, the ideal concentration was

248 established for dNTPs at 200 µM (each dNTP) and for Taq polymerase at 1.25 U/15 µl of
249 reaction (Table 2).
250
< Table 2
251 Finally, the two gel staining protocols with GelRed™ showed important differences. For
252 instance, precasting gel staining was more sensitive to stain DNA but migration in
253 electrophoresis was interfered, producing curved or wrinkled bands which complicate gel
254 interpretation. Conversely, although it is a slightly longer process, post staining gel
255 produced clear and straight bands.
256
257 *Detection and removal of PCR-inhibitors*
258 It was possible to identify that any of the evaluated DNA extraction methods remove PCR-
259 inhibition agents consistently, due to percentages of successful amplification were
260 deficient: 50% for DNA extracted by PCI, 45% for salt-extracted DNA and 54% for
261 Promega Kit extractions. Verification tests demonstrated the occurrence of PCR-inhibitors
262 within *S. gigas* DNA samples that did not amplify, since reactions with the mixture of DNAs
263 (butterfly and suspect queen conch) also showed absence of products (Fig. 2). Concerning
264 dilution tests, results showed that PCR efficiency was increased only with extracts diluted
265 at 1:5, allowing suitable amplification of around 35% of the samples that had shown
266 inhibition problems. For assays with DNA extracts in 1:15 and 1:30 dilutions, the presence
267 of smears and faint bands suggest that amplification was performed; however, bands
268 rarely exposed enough intensity and sharpness for their appropriate identification.
269 Although it represents a considerable loss of the extracted DNA (65% on average) and an
270 increase in processing cost (US $1.85 per sample), the usage of purification columns was
271 the best strategy to remove undesirable compounds within *S. gigas* DNA extracts,
272 attaining proper amplification in ca. 80% of 'problematic' samples.

< Figure 2

< Figure 3

DISCUSSION

As DNA extraction is a critical and expensive first stage in genetic research, it is a common target for optimization (Ivanova, Dewaard & Hebert, 2006). To date, different protocols have been used to obtain DNA from *S. gigas*, particularly through the use of commercial kits (Morales and Lopez, 2003; Zamora-Bustillos *et al*., 2011; Márquez *et al*., 2013), and methods which employ phenol-chloroform (Morales y Lopez, 2003) or CTAB (Pérez-Enriquez *et al*., 2011) as key reagents in the procedure. One downside of most commercial kits is the high cost (e.g. US $3.45/sample with DNeasy Blood & Tissue Kit of Qiagen) and that sometimes require highly specialized equipment (e.g. QuickGene DNA Tissue kit of Fujifilm). The drawback of phenol-chloroform methods, is that reagents are physically, healthy and environmentally hazardous; while the employment of CTAB often results in DNA degradation (Fang, Hammar & Grumet, 1992).

In this study we analyzed the trade-offs of three DNA extraction protocols, which are based on different DNA isolation principles. For example, PCI procedure is based on the KCl precipitation of polysaccharides followed by protein elimination with phenol-chloroform-isoamylic alcohol, whereas salt extraction and Promega Kit methods involve the *salting out* technique to isolate genomic DNA from a wide variety of undesirable compounds.

Our findings indicate that the PCI method provides satisfactory results for *S. gigas* in terms of quantity and integrity of DNA. However, although this protocol is specifically reported to overcome problems associated with the DNA extraction of mollusk tissues (Sokolov, 2000), DNA efficiency in subsequent PCR amplifications was variable. Because the PCI method includes major steps to remove polysaccharides and proteins, PCR-inhibition in these samples could be attributed to phenol residues in DNA extracts, as suggested by Popa *et al*. (2007). Nevertheless, in the present study we were unable to carry out specific tests to confirm this. Furthermore, despite PCI is a moderate-cost method, it has the disadvantage of employing hazardous reagents and its implementation is considerably laborious.

The salt extraction method is referred as a universal and rapid DNA extraction protocol (Aljanabi & Martinez, 1997). Here we demonstrated its applicability to obtain suitable amounts of HMW DNA from *S. gigas* foot muscle. Although the *salting out* technique has been applied to obtain PCR-able DNA from cephalopods (Garoia *et al*., 2004), it is not widely mentioned in literature for DNA extraction in mollusks. For the queen conch we obtained partially satisfactory results: less than half of DNA extracts obtained using this protocol presented successful amplification. Contrary to PCI, this procedure requires no toxic chemicals, is low cost and is simple to perform.

The total amount of DNA per sample obtained with the Promega extraction Kit was approximately 1.5 times lower when compared with the rest of the evaluated protocols. Nevertheless, DNA integrity remained similar to that attained with PCI and salt extraction methods. Positive aspects of this method are that it avoids the use of hazardous chemicals (and concerns of accompanying waste disposal) and is quicker to perform, but is 5.6 and 2.4 times more expensive to carry out than the salt extraction protocol or PCI method respectively. In addition, we found that PCR-inhibitory agents were present in almost half of the samples processed with the Promega extraction Kit; however, Zamora-Bustillos*, et al.* (2011) have used this commercial system to obtain PCR-able DNA from the foot

muscle of *S. gigas*, without mentioning PCR inhibition problems for microsatellite amplifications.

A relevant aspect found in this study is that the presence of PCR-inhibitors within the DNA extracts was strongly related to the place of origin of samples. Analyzing the information obtained with the salt extraction protocol, it was possible to observe that DNA from individuals sampled at CPAFF show low rates of PCR inhibition (~0.06). This means that the majority of those samples did not require dilutions or purification columns for their successful amplification. In contrast, rates of PCR inhibition were evidently greater for DNAs obtained from PMNRP (~0.74), XNRP (>0.95) and BCBR individuals (>0.95).

There is no clear explanation for this. A valid argument is the possibility of inherent differences in samples, specifically in their content of PCR-inhibitory substances. Garr *et al.* (2011) noted that the protein and polysaccharide content in *S. gigas* meat changes significantly according to diet composition, even over relatively short periods of time (24 weeks). Taking this into consideration it is possible (depending on the place of origin of our samples) that sufficient amounts of proteins and/or polysaccharides were present in our DNA source tissue, to partially remain in some samples after DNA extraction and thus affect the PCR. This argument could also explain the differences of PCR inhibition between the present study and the research conducted by Zamora-Bustillos*, et al.* (2011) using the Promega extraction Kit.

An alternative explanation for these differences could be a higher sensitivity of ISSR primers to PCR inhibitors than microsatellites. If correct, the mechanism of inhibitor action in our samples should be thorough the interference of primer annealing to template, and primers with high melting temperatures would be able to overcome inhibition (Opel, Chung & McCord, 2010). During the adaptation of the ISSR technique, an absence of amplification was observed in some samples using useful ISSR primers with elevated *Tm*, such as $(GAG)_5GC$ (data not shown). Márquez (pers. comm.) also observed amplification problems with DNA samples of *S. gigas* obtained using the Promega Kit and microsatellites as molecular system. Thus, higher sensitivity of ISSR to PCR inhibitors appears to be unlikely. Other potential explanations remain less clear, considering that tissue samples in the present work were stored and processed as in Zamora-Bustillos*, et al.* (2011). Regardless, it would be necessary to develop focused studies in order to clarify these spatial and temporal inhibition differences.

A variety of techniques have been applied to relieve PCR-inhibition: dilution of the DNA sample or increase of polymerase and magnesium concentrations, or even more complex DNA extractions and cleanup stages (Opel*, et al.*, 2010). For *Strombus gigas* none of the evaluated DNA extraction protocols were able to uniformly eliminate agents which impede the PCR. Sample dilution was a simple course of action to reduce inhibitors within DNA extracts. However, its effectiveness was only observed in some 5-fold diluted samples. This amplification deficiency is attributable to the fact that this method does not consider - for example- sample variability of target DNA and inhibitor concentrations, making it too unreliable for field samples (Kosch & Summers, 2013). In contrast, the bind-resin principle of Promega purification columns demonstrated to be a cost-effective tool for the removal of unwanted compounds within *S. gigas* DNA extracts, with the drawback of considerable DNA loss, also reported by Harnpicharnchai *et al*. (2007). However, we can consider that such loss represents a limitation only for samples where the total DNA amount is reduced.

As other molecular tools, ISSR requires particular PCR conditions to improve its performance. We found that ISSR primers can anneal template DNA over a relative wide temperature interval (~4°C) without considerably affecting the process, because stringent annealing temperatures, primer lengths and low primer-template mismatch (as result of anchoring) favor ISSR reproducibility (Abbot, 2001). For its part, concentration of template DNA presented improved amplification results using similar values to those frequently reported in ISSR literature (between 10 and 30 ng of template DNA per ~20 µl of mix reaction). However, the occasional inhibition in reactions containing larger amounts of DNA is not attributed to high quantities of template DNA *per se*, but to the possibility of a major addition of inhibitory substances present in DNA extracts, since the use of higher quantities of template DNA has been reported without amplification interference (e.g. Hou *et al*., 2006).

Differences in PCR performance have been identified when variable concentrations of $MgCl_2$, primer, dNTPs and Taq are used (Bornet & Branchard, 2001). Our results are partially consistent with this, because the variation of intensity and number of amplified products was observed only when $MgCl_2$ and primer concentrations were modified. A possible explanation for the apparent null effect of variation in the concentration of dNTPs is that we evaluated a relative reduced interval (from 200 to 400 µM each dNTP) compared with other studies (from 20 to 700 µM each dNTP; Bornet and Branchard, 2001).

Analogue to DNA extraction, the election of a molecular tool is critical for the appropriate development of genetic studies. This mainly depends on the objectives of the study, as well as the pragmatics of reproducibility, time availability and costs of technique materials (Bornet & Branchard, 2001; Parker *et al.*, 1998). Molecular genetic analysis focused on wildlife monitoring and conservation issues requires a large set of reliable, highly informative and cost effective genetic markers (Coupé *et al.*, 2011), hence ISSR characteristics are of particular interest for research on endangered species. The attractiveness this technique adapted for *S. gigas* is the increased reproducibility and reliability of its results (through the detection of several polymorphic *loci*), as well as a performance that allows the development of high resolution genetic studies even with very small amounts of DNA source tissues (Casu *et al.*, 2008; Moraga-Suazo *et al.*, 2012), thus avoiding the use of lethal sampling methods. In addition, although co-dominant markers are more informative per *locus* (Moraga-Suazo*, et al.*, 2012), the identification of a set of functional ISSR primers for *S. gigas* in this study and their multi-*loci* characteristics favors robust genetic profiles (Fig. 3), opening the way for future research aimed at clarifying the population structure of this threatened species (at the local and wider geographical scales).

In summary, the results of this study suggest the employment of the salt extraction method proposed by Aljanabi & Martinez (1997), with the additional usage of purification columns (Wizard® DNA Clean-Up System, Promega) on DNA extracts, as an appropriate route to assure the extraction of PCR-able DNA from the queen conch. This work also confirms ISSR functionality on *S. gigas*, an attractive alternative for further research that contributes to resource management and conservation.

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FIGURE CAPTIONS

Figure 1. Electrophoresis of extracted DNA using three different protocols. A. Phenol/chloroform/isoamylic alcohol. B. Salt extraction. C. Promega Kit. L. 100 bp DNA ladder. Arrow indicates high molecular weight DNA.

Figure 2. Verification of the presence of PCR-inhibitors in two different *S. gigas* DNA extracts using the BDB$(ACA)_5$ primer and *Ta* = 57 °C. Reactions contained only butterfly DNA as template (CR), only queen conch DNA (1 and 2) or butterfly DNA plus queen conch DNA (3 and 4). L. 100 bp DNA ladder. This image shows exclusively *S. gigas* DNA from Promega extraction kit (tests for PCI and salt extraction DNA revealed the same inhibition pattern).

Figure 3. Example of polymorphic ISSR banding patterns produced by the primer $(GAG)_5GC$ in *S. gigas*. Samples from CPAFF (1 and 2), PMNRP (3 and 4), BCBR (5 and 6) and XNRP (7 and 8) were employed. DNA was obtained by salt extraction and afterwards purified with the Wizard® DNA Clean-Up System. L. 100 bp DNA ladder. Refer to methods section for abbreviation of collection sites.

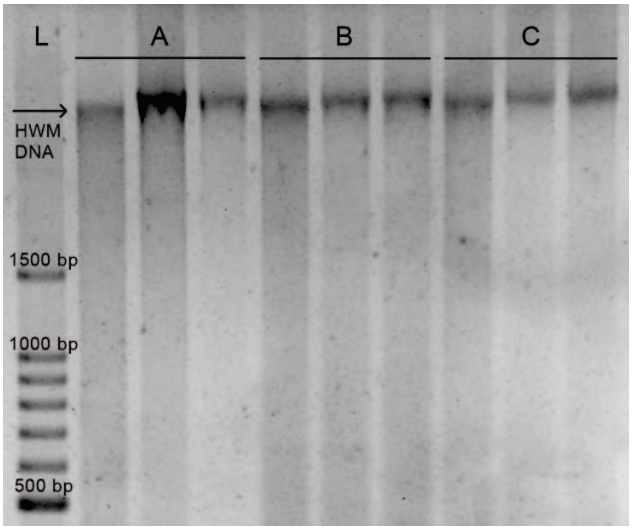
619 TABLES
620
621
622 **Table 1.** Attributes of ISSR primers tested by PCR amplification in *Strombus gigas*. GC:
623 guanine-cytosine content; *Tm*: melting temperature; *Ta*: annealing temperature; NB:
624 number of scored bands. B = C, G or T; D = A, G or T; R = A or G; W = A or T; Y = C or T.
625

Sequence (5'→ 3')	GC (%)	*Tm* (°C)	*Ta* (°C)	Banding resolution	NB	Size range of bands (bp)
$(CA)_7$	50	55.2	54	Absence of amplification	-	-
$(CT)_8C$	52.9	55.7	54	Absence of amplification	-	-
$(GA)_8G$	52.9	55.7	54	Absence of amplification	-	-
$(GAA)_6$	33.3	53.9	52	Poor amplification	-	-
$(GA)_8C$	52.9	56	54	Poor amplification	-	-
$(GACA)_4$	50	57.1	56	Poor amplification	-	-
$(AC)_8G$	52.9	62.5	61	Poor amplification	-	-
$BDB(ACA)_5$	37	58.4	57	Smeared	-	-
$WB(GACA)_4$	48.1	61.5	60	Smeared	-	-
$(ACA)_5BDB$	37	58.6	57	Smeared with bands	-	-
$(CA)_8AC$	50	61.4	60	Smeared with bands	-	-
$(AC)_8C$	52.9	62.3	61	Smeared with bands	-	-
$(AG)_8G$	52.9	57.5	56	Suitable for scoring	6	200-600
$(AG)_8Y$	50	57.6	56	Suitable for scoring	4	200-600
$(AG)_8C$	52.9	58.2	57	Suitable for scoring	4	200-600
$(GACA)_4WB$	48.1	59	57	Suitable for scoring	22	200-1300
$(AG)_8YC$	52.8	59.5	58	Suitable for scoring	26	100-1400
$(GT)_8C$	52.9	60.4	59	Suitable for scoring	5	400-900
$(CA)_8AG$	50	61.1	60	Suitable for scoring	19	200-1400
$(CA)_8GT$	50	62.5	61	Suitable for scoring	9	200-1100
$(CA)_8RY$	50	62.1	61	Suitable for scoring	8	400-700
$(GAG)_5GC$	70.6	64.8	63	Suitable for scoring	28	200-2000
$(GTG)_5GC$	70.6	67.8	66	Suitable for scoring	27	100-1500

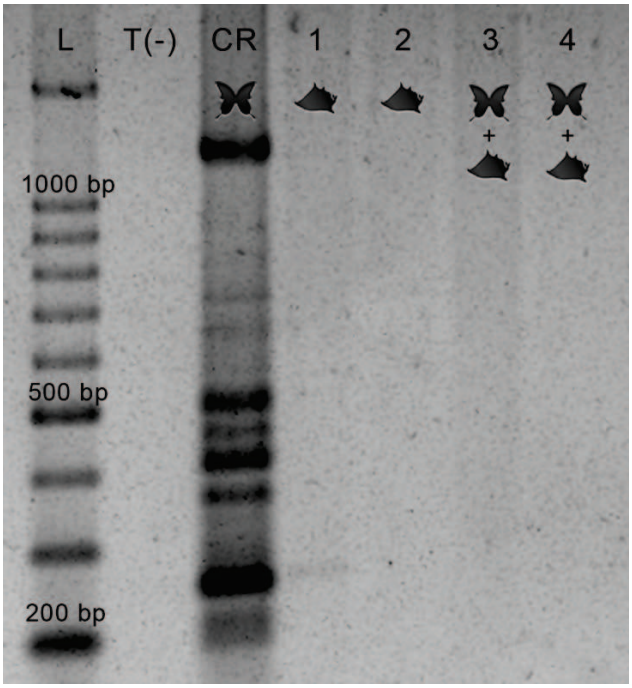
626

Table 2. Banding resolution for varied amounts/concentrations of different PCR parameters (reaction volume = 15 µl). ++: Excellent; +: Good; -: Unsuitable.

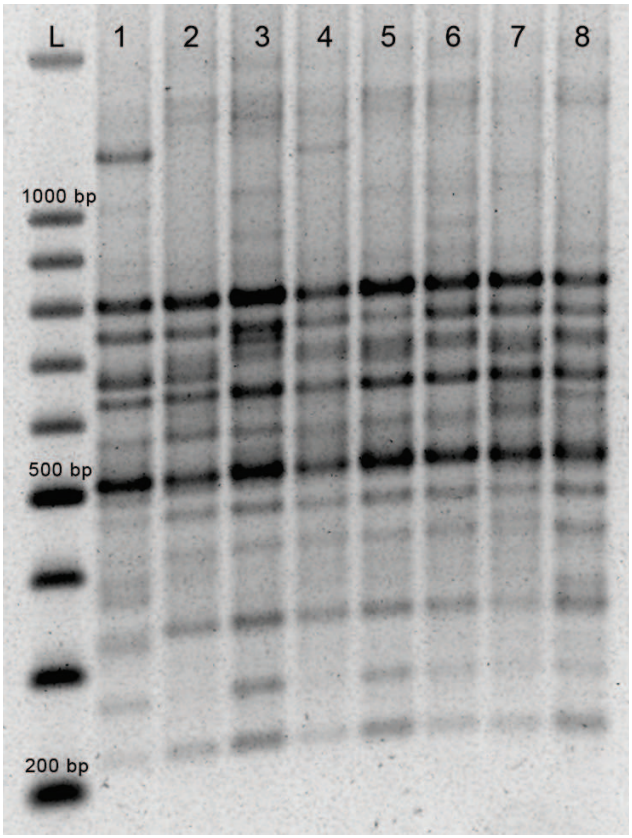
Template DNA		*$MgCl_2$*			*Primer*			*dNTPs (each)*		*Taq polymerase*		
6-25 ng	25-200 ng	2 mM	3 mM	4 mM	0.5 µM	1 µM	2 µM	200 µM	400 µM	1 U	1.5 U	2 U
++	+	-	++	-	-	++	-	+	+	+	+	+



631 Figure 1



632 Figure 2



633 Figure 3

Conclusiones

La extracción de ADN es una etapa inicial fundamental para el adecuado desarrollo de investigaciones genéticas. En este estudio se analizaron las ventajas y desventajas de tres diferentes protocolos para la extracción de ADN a partir de músculo del pie de *S. gigas*: el método de fenol-cloroformo-alcohol isoamílico (PCI), el protocolo de extracción por sales y el uso del Kit comercial de Promega. El protocolo de extracción por sales presenta ventajas en términos de costo e inocuidad para el usuario/ambiente. Los tres métodos evaluados producen cantidades adecuadas de ADN de alto peso molecular; sin embargo, la funcionalidad de los diferentes extractos de ADN en la PCR fue inconsistente. Pudo demostrarse que la inhibición en la PCR es causada por sustancias contenidas en los extractos de ADN, lo que sugiere 1) ineficiencia del protocolo para remover consistentemente sustancias inhibidoras y/o 2) contaminación por residuos de reactivos durante el procesamiento de muestras (protocolo PCI).

Un aspecto relevante encontrado en esta investigación, fue la presencia de agentes inhibidores de la PCR en relación con la localidad de procedencia del material biológico. Aunque no existe una clara explicación para esto, el análisis de la información existente sugiere la posibilidad de diferencias inherentes a las muestras biológicas, particularmente en su contenido de sustancias que inhiben la PCR (e.g. proteínas y polisacáridos). Como resultado de la falta de información específica, otras potenciales explicaciones permanecen inciertas. En todo caso, sería necesario desarrollar estudios

puntuales para esclarecer estas diferencias, ya que fenómenos similares no son reportados con frecuencia en la literatura.

A pesar de que algunos estudios han empleado esta estrategia para superar la inhibición en la PCR, la dilución de los extractos de ADN es un método poco efectivo cuando la concentración de ADN y/o inhibidor es variable entre muestras. Contrariamente, el uso de columnas de afinidad para la purificación de ADN de caracol rosado, es una estrategia adecuada para eliminar agentes que impiden la amplificación de secuencias. En virtud de lo anterior, se sugiere el uso del protocolo de extracción por sales -con la adicional purificación de ADN mediante columnas de afinidad- como la ruta más sencilla, económica y efectiva para la obtención de ADN funcional a partir de músculo del pie de *S. gigas*.

Adicionalmente, en este trabajo se confirmó la aplicabilidad de la técnica ISSR para *S. gigas*, obteniendo un conjunto de 11 marcadores funcionales para la especie. De manera general, los resultados de este estudio denotan la plasticidad de los ISSR, ya que: 1) la hibridación con el ADN molde pudo efectuarse dentro de un amplio intervalo de temperaturas, sin afectar considerablemente el proceso de amplificación; 2) el uso diferentes cantidades de ADN molde en la reacción no interfiere con su amplificación (aunque se sugiere el uso de una cantidad uniforme en todas las reacciones para obtener resultados constantes); y 3) distintos marcadores comparten las mismas condiciones de amplificación (e.g. concentraciones de $MgCl_2$/dNTPs/Taq polimerasa), lo que simplifica el proceso de adaptación de esta herramienta.

Al igual que la extracción de ADN, la elección de una herramienta molecular es esencial para el adecuado curso de una investigación genética. Estudios de genética poblacional que emplean ISSR, requieren del análisis del mayor número de *loci* posible (comúnmente >80). El atractivo particular de haber identificado 11 marcadores ISSR para el caracol rosado, reside en que estos permitieron el reconocimiento de un gran número de diferentes *loci* (visualizados como bandas), lo que exhibe el potencial de esta herramienta molecular para obtener información genética confiable y con elevado nivel de resolución. Asimismo, su alto rendimiento le confiere a los ISSR la cualidad de desarrollar análisis genéticos incluso empleando pequeñas cantidades de tejidos fuente de ADN, lo cual evita el uso de técnicas letales de muestreo.

Este estudio es una contribución inicial de particular relevancia para la mejora del manejo y conservación del caracol rosado. En él, se presentan las bases técnicas para la obtención de información molecular de *S. gigas*, facilitando el desarrollo de investigaciones futuras que analicen su estructura poblacional y diversidad genéticas sobre diferentes extensiones geográficas y ventanas de tiempo (monitoreo genético).

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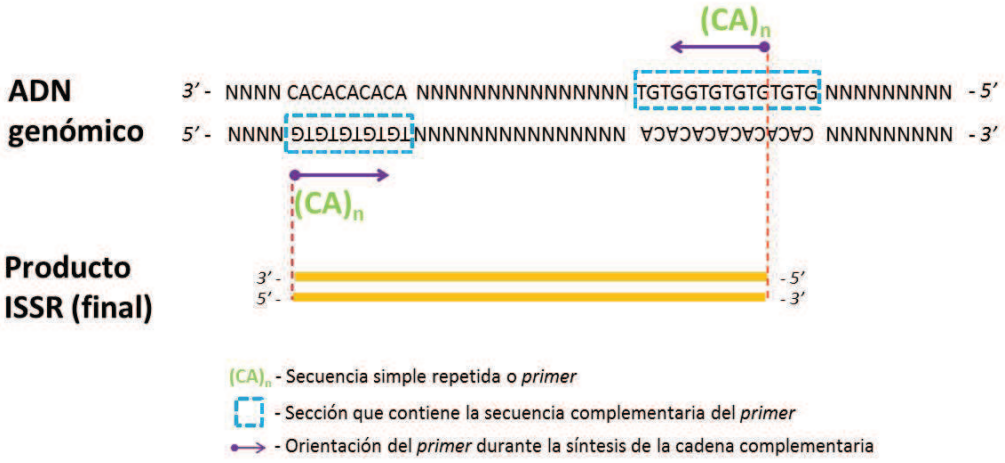
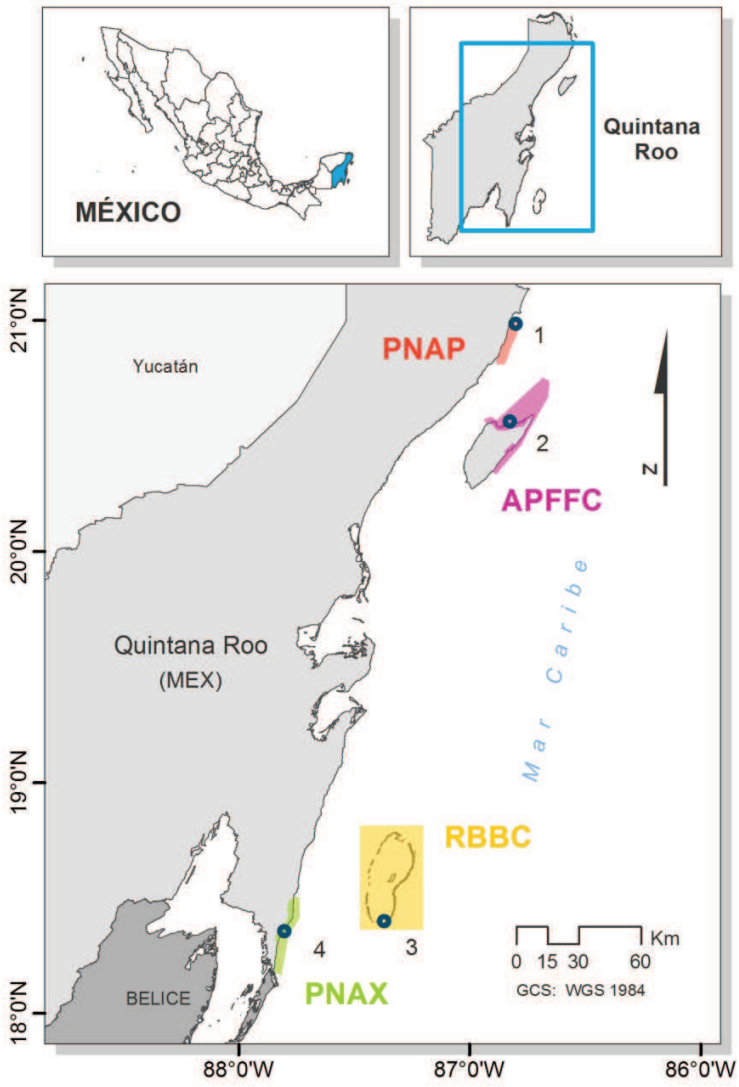
Anexos

1. Representación básica del funcionamiento de la herramienta molecular ISSR (modificada de Zietkiewicz et al., 1994).



Nota: La amplificación simultánea de múltiples regiones ISSR depende de la presencia y proximidad de otras secciones –orientadas inversamente- que contengan la secuencia complementaria del *primer*.

2. Sitios de colecta del material biológico empleado en este estudio (puntos). Los polígonos representan diferentes áreas naturales protegidas. PNAP: Parque Nacional Arrecifes de Puerto Morelos; APFFC: Área de Protección de Flora y Fauna Isla de Cozumel; RBBC: Reserva de la Biósfera Banco Chinchorro; PNAX: Parque Nacional Arrecifes de Xcalak.



3. Permiso de pesca de fomento

DGOPA -PF -01

ESTADOS UNIDOS MEXICANOS

SECRETARÍA DE AGRICULTURA, GANADERÍA, DESARROLLO RURAL, PESCA Y ALIMENTACIÓN
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PERMISO DE PESCA DE FOMENTO

FOLIO No. 064/13

Permiso de Pesca de Fomento No. PPF/DGOPA-064/13 **Vigencia del** 11 de junio de 2013 **Al** 10 de junio de 2014

Expedido en: Mazatlán, Sinaloa El dia 6 de junio de 2013

Titular del Permiso: El Colegio de la Frontera Sur (ECOSUR-Unidad Chetumal) **Clave R.N.P.**

Domicilio: Ave. Centenario km. 5.5 S/N **C.P.** 77014

Localidad: Chetumal **Municipio:** Othón P. Blanco **Entidad:** Quintana Roo

Título del Proyecto: "Caracterización molecular de poblaciones del caracol rosado *Strombus gigas* en el Sur de Quintana Roo".

Zona de Operación: Aguas marinas de jurisdicción federal del Golfo de México, específicamente en el sur del Estado de Quintana Roo (Cayo norte y Cayo lobos en la Reserva de la Biósfera de Banco Chinchorro y Punta Gavilán en el Parque Nacional Arrecifes de Xcalak).

Nombre(s) de la(s) Embarcación(es) Autorizadas:	Número de Matrícula(s):	Eslora (MTS):
1.- COBIA	2306075514-2	7.910

Artes o Equipos de Pesca Autorizados:

-Equipo de buceo autónomo, membrana de fibra de vidrio Millipore de 45 µm, etanol 96%, NaCl al 0.9% y 1 mM $EDTANA_3$, tubos de plástico.

Sitio de Desembarque Autorizado o Puerto Base: Xcalak, Quintana Roo

Nombre del Investigador Responsable: Dra. Salma Christine Machkour M'Rabet.
Institución que respalde SI [x] **No** [ ] **Nombre:** El Colegio de la Frontera Sur (ECOSUR-Unidad Chetumal)

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2.-Este permiso se concede sin perjuicio de los permisos o autorizaciones que requieran de otras autoridades competentes.
3.-Los documentos nacionales o extranjeros que se publiquen como resultado de las actividades realizadas, deberán hacer referencia al número del permiso correspondiente otorgado por esta Comisión.
4.-El presente permiso y sus efectos, se extinguirán por cualquiera de las causas señaladas en los artículos 52, 53, 54, 55, 56 y 57 de la Ley General de Pesca y Acuacultura Sustentables o por incumplimiento de las obligaciones que le impone el mismo.

Con fundamento en lo dispuesto en los artículos 1°, 2°, fracción X y XV, 4° fracción XXXII y 28 fracción II, III, VI, 41 fracción V y 64 de la Ley General de Pesca y Acuacultura Sustentables; 16 fracciones I y II, 20, 25, 29, 30 fracción I inciso b), 31 fracción II inciso c), 33, 68, 71, 72, 74,76 y 78 de su Reglamento; artículo 35 fracciones XXI y XXII de la Ley Orgánica de la Administración Pública Federal; artículos 1°, 2° inciso d), fracción III y octavo transitorio del Reglamento Interior de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación.

AUTORIDAD EXPEDIDORA

LIC. ALDO GERARDO PADILLA PESTAÑO	DIRECTOR GENERAL		HOJA 1 DE 3
NOMBRE	CARGO	FIRMA	

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ESTADOS UNIDOS MEXICANOS

DGOPA -PF -02

SECRETARÍA DE AGRICULTURA, GANADERÍA, DESARROLLO RURAL, PESCA Y ALIMENTACIÓN
COMISIÓN NACIONAL DE ACUACULTURA Y PESCA
DIRECCIÓN GENERAL DE ORDENAMIENTO PESQUERO Y ACUÍCOLA
PERMISO DE PESCA DE FOMENTO

FOLIO No. 064/13

TERMINOS Y CONDICIONES:

1.- Objetivo General:

I. Caracterización molecular de poblaciones del caracol rosado *(Strombus gigas)* en el Sur de Quintana Roo, utilizando marcadores ISSR.

2.- Objetivos Particulares:

I. Identificar los mejores marcadores ISSR, para la especie *S. gigas*.
II. Determinar la diversidad genética de cada población en estudio y evaluar el grado de conectividad existente entre ellas.
III. Evaluar el potencial del uso de ADN proveniente del mucus del pie del caracol rosado, determinando la idoneidad de ésta técnica no invasiva con fines moleculares.

3.- Participantes:

Dra. Salima Christine Machkour M'Rabet Investigadora Titular A del Colegio de la Frontera Sur (Unidad Chetumal), quien acreditó su capacidad científico-técnica conforme lo establece en el artículo 72 del Reglamento de la Ley de Pesca en colaboración con el C. Artemio Venancio Poot Balam y personal designado por la responsable del proyecto.

4.- En campo se contemplan las siguientes actividades:

I. La colecta de caracol rosado *Strombus gigas* mediante buceo autónomo en tres zonas al Sur de Quintana Roo: Cayo norte y Cayo lobos en la Reserva de la Biósfera de Banco Chinchorro y Punta Gavilán en el Parque Nacional Arrecifes de Xcalak.
II. Realizar la colecta de muestras a profundidades entre los 5 y 12 m.
III. En cada sitio se examinarán 35 ejemplares de caracol rosado, dando un total de 105 organismos, de los que se obtendrá aproximadamente 1g. del músculo del pie mediante una escisión.
IV. La colecta de mucus de 10 organismos por sitio, realizando un lavado de los caracoles con agua de mar filtrada (a través de una membrana de fibra de vidrio *Millipore* de 45 µm) para evitar contaminación cruzada de ADN. Después se removerá el mucus utilizando un hisopo humedecido con una solución compuesta por NaCl al 0.9% y 1 mm de $EDTANa_2$, mismo que es sumergido en 1 ml de la misma solución contenida en un tubo de plástico, al cual se añaden 9 ml de etanol al 96%.
V. La técnica para realizar el estudio será no invasiva (no sacrificar el organismo). Posterior a la toma de muestra, cada caracol será liberado en su lugar de captura.
VI. Las muestras de tejidos y mucus serán correctamente etiquetadas y almacenadas a baja temperatura para su traslado al laboratorio, donde se pondrán en refrigeración a 4 °C para su posterior procesamiento.

5.- El responsable del Proyecto queda obligado a:

I. El calendario de colecta deberá ser modificaco de manera que se especifique una fecha tentativa de inicio de los muestreos, misma que podrá ser a partir del otorgamiento del permiso de pesca de fomento hasta la fecha de fin de vigencia.
II. Las estaciones de muestreo se encuentran dentro de un Área Natural Protegida, por lo que se deberán realizar los trámites correspondientes ante la CONANP de la SEMARNAT.
III. Los procedimientos de la colecta deberán cumplir con lo establecido en la NOM-126-SEMARNAT-2000, que establece las especificaciones para la realización de actividades de colecta científica de material biológico de especies de flora y fauna silvestres.
IV. Los colectores deben tener cuidado de no dañar los organismos colectados y regresarlos al medio con la menor manipulación posible.
V. Todas las actividades de muestreo deberán quedar registradas en las bitácoras de campo que incluya la referencia geográfica del lugar de la toma de materia biológico.
VI. En el caso de las publicaciones o ponencias en foros de índole científico o de divulgación, se deberá hacer la referencia que la información es producto de un permiso de pesca de fomento.

AUTORIDAD EXPEDIDORA

LIC. ALDO GERARDO PADILLA PESTAÑO	DIRECTOR GENERAL	
NOMBRE	CARGO	FIRMA

HOJA 2 DE 3

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INTERESADO

ESTADOS UNIDOS MEXICANOS

DGOPA -PF -02

SECRETARÍA DE AGRICULTURA, GANADERÍA, DESARROLLO RURAL, PESCA Y ALIMENTACIÓN

COMISIÓN NACIONAL DE ACUACULTURA Y PESCA

DIRECCIÓN GENERAL DE ORDENAMIENTO PESQUERO Y ACUÍCOLA

PERMISO DE PESCA DE FOMENTO

FOLIO No. 064/13

TÉRMINOS Y CONDICIONES (CONTINUACIÓN):

VII. Al término del proyecto y como resultado de los trabajos de investigación, se deberá entregar a esta Dirección General de Ordenamiento Pesquero y Acuícola, el informe final respectivo y de las publicaciones producto de la autorización del permiso de pesca de fomento y éste deberá tener la formalidad que se debe cubrir en la formulación de un informe de investigación.

VIII. Conservar el presente permiso durante las operaciones de campo y mostrarlo a las autoridades competentes que se lo soliciten.

IX. Permitir y facilitar las tareas de inspección que en su caso llegaran a realizar las autoridades competentes, lo anterior en términos de los artículos 128 y 129 de la Ley General de Pesca y Acuacultura Sustentables, apercibido de que de no hacerlo, se procederá a la revocación del permiso en términos del artículo 55 fracción II de la misma Ley.

X. Deberá llevar a bordo de la embarcación menor autorizada, el original o copia certificada del presente permiso con firma y sello autógrafo de la Subdelegación de Pesca del Estado.

5.- Queda estrictamente prohibido:

I. El empleo de cualquier tipo de explosivos o de sustancias tóxicas.

II. Efectuar colecta, transporte y aprovechamiento alguno de las especies de flora y fauna silvestre, raras, amenazadas y el peligro de extinción, incluidas en la Norma Oficial Mexicana (NOM-059-SEMARNAT-2010) publicada en el Diario Oficial de la Federación el 30 de Diciembre de 2010.

III. El uso de redes arrastre en bahías y esteros.

IV. Realizar captura con fines de comercialización.

AUTORIDAD EXPEDIDORA

LIC. ALDO GERARDO PADILLA PESTAÑO — NOMBRE

DIRECTOR GENERAL — CARGO

FIRMA

HOJA 3 DE 3

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INTERESADO