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OPCIÓN EN CONSERVACIÓN Y MANEJO DE RECURSOS NATURALES

FILOGEOGRAFÍA Y CARACTERIZACIÓN DEL TINTE DEL CARACOL PÚRPURA *Plicopurpura pansa* (Gould 1853)

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A:

M. C. FELIPE JAVIER LÓPEZ CHÁVEZ

DIRECTOR DE TESIS: DRA. PATRICIA RÍOS CHÁVEZ

CO-DIRECTOR DE TESIS: DR. ALBERTO KEN OYAMA NAKAGAWA

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El presente trabajo se realizó en los laboratorios de Fitobioquímica de la Facultad de Biología de la Universidad Michoacana de San Nicolás de Hidalgo y de Ecología Genética y Molecular de la Universidad Autónoma de México, campus Morelia, bajo las asesorías de la Dra. Patricia Ríos Chávez y del Dr. Ken Oyama Nakagawa

ÍNDICE

Resumen

El caracol púrpura (*Plicopurpura pansa,* Gould 1853) ha sido un recurso importante desde tiempos precolombinos por su uso para teñir prendas ceremoniales, entre otros usos. En México se prohibió el uso del caracol para extraer el tinte en la década de los 80's a causa de un mal manejo del recurso que llevó a las poblaciones a disminuir su tamaño poblacional y la talla de los organismos. El gobierno mexicano declaró el recurso como sujeto de protección especial y solo los mixtecos de Pinotepa de Don Luis, en Oaxaca, tienen permiso para su explotación. Esto ha originado la necesidad de conocer la biología y ecología de la especie para un manejo sustentable. En el presente trabajo se estudiaron los metabolitos volátiles que acompañan a los precursores del colorante 6,6'-dibromoíndigo en la glándula hipobranquial. Se utilizó la técnica de cromatografía de gases acoplada a espectrometría de masas. Se identificaron cuatro compuestos conocidos y once compuestos, no identificados, derivados del indol en *Plicopurpura pansa*, mientras que las especies *P. columellaris* y *P. patula* presentan un compuesto compartido con *P. pansa,* el tirindoleninona y cada una de ellas presenta un compuesto único. Por otro lado, se analizó la variabilidad genética de las poblaciones de *P. pansa* a lo largo del Pacífico Mexicano para conocer la estructura filogeográfica, esto con la ayuda de secuencias del gen mitocondrial del citocromo b. Las poblaciones mexicanas muestran una diversidad haplotípica alta con un promedio de 0.765 y una diversidad nucleotídica baja, con un promedio de 0.0045, que de acuerdo con algunos autores estos valores representan poblaciones de reciente creación o que se están recuperando de un cuello de botella. Estos resultados son similares con los de otros moluscos gasterópodos habitantes de la zona intermareal rocosa, ya que en la bibliografía revisada estos organismos presentan valores altos de diversidad haplotípica y valores bajos de diversidad nucleotídica (Tabla 2 del capítulo 2), estos organismos, aparte de ser intermareales rocosos presentan, la mayoría, una etapa larvaria de vida libre al igual que *P. pansa*, estas similitudes se dan en extensiones como las presentadas en el presente trabajo. Un análisis de red de haplotipos en donde encontramos una conformación de estrella, indicio de poblaciones en

expansión. Por último obtuvimos en un análisis de distribución “mismatch” una curva que se ajusta al modelo de expansión, resultado que confirma lo mostrado por el estadístico Fs de Fu, que muestra en todas las poblaciones un resultado negativo y significativo lo cual indica que son poblaciones en expansión o recuperándose de un cuello de botella.

Abstract

Purple snail (*Plicpurpura pansa* Gould 1853) has been an important resource since pre-Columbus times due to the dye that is still used on ceremonial clothes among other things. Back in the ‘80s there was an irrational extraction of the dye so the populations diminished in numbers losing the bigger snails as in the organism size. The Mexican government placed *P. pansa* as a special concern species so the resource use is restricted and managed by the Mixtecan people from Pinotepa de Don Luis in Oaxaca state. This situation has led researchers to study the biology and ecology of *P. pansa* for a sustainable use. In the present work the volatile compounds from the hypobranchial gland that come out along with the 6,6’-dibromoindigo precursors of the organisms were isolated. The follow up was accomplished by the gas chromatography coupled to mass spectrometry analysis. As result, four known indole derivative compounds and eleven unknown indole derivatives were found. The tyrindoleninone was present in all three species of *Plicopurpura*. *P. patula* and *P. columellaris,* each one of the species presents a unique volatile indole derivative compound and other compounds were presented in both of them but not in *P. pansa*. On the other hand, the population genetic structure of *P. pansa* along the Mexican Pacific shore was analyzed to determine the phylogeographic structures, this with the aid of sequences of the mitochondrial citochrome b gene. The results obtained display that the populations are of recent creation, as the haplotype diversity (h) was high for the Mexican populations with a mean of 0.765 and low nucleotide diversity (π) with a mean of 0.0045. This combination, according to some authors, corresponds to populations that are expanding or recovering from a bottleneck. These values of high haplotype diversity and low nucleotide diversity are similar to some rocky intertidal gastropods since they have high h and low π (see table 2 in chapter 2) apart from being rocky intertidal inhabitants most of these species have a planktonic larva. The haplotype network is a starlike politomy, and that is another way of seeing expanding populations or recovering from a bottleneck. Mismatch distribution analysis is the third evidence that concludes the same as the former two, the data presented herein match with expanding population model, and this mismatch

distribution analysis also confirms the Fu's statistic which were all negative for all populations and that means that populations are recovering from a bottleneck.

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

I. Introducción

EL caracol púrpura (*Plicopurpura pansa*) es un gasterópodo habitante de la zona intermareal rocosa del Pacífico tropical oriental. El género *Plicopurpura* presenta tres especies las cuales son habitantes de la zona intermareal rocosa, encontrándose *P. pansa* y *P. columellaris* en la costa del Pacífico y *P. patula* en la del Atlántico. Estas tres especies estaban ubicadas dentro del género *Purpura* pero en 1988 Kool propuso que existe evidencia suficiente para ubicar a estas tres especies en un género diferente, por lo que recomienda retomar el género *Plicopurpura* propuesto por Cossmann en 1908 y a partir de ese momento se les ubica en este género.

El caracol púrpura *P. pansa* era considerado una subespecie de *P. columellaris* (Wellington y Kuris 1983) y también se le ha considerado como subespecie de *P. patula* (Kool 1988, Skoglound 1992). En 1992, Castillo-Rodríguez determina separar a *P. pansa* como especie, con base en las características morfológicas de la rádula y del tracto digestivo. El caracol púrpura *P. pansa* es un depredador de la zona intermareal rocosa a lo largo de la costa del Pacífico oriental encontrándose desde el sur de Baja California Sur hasta el norte de Perú incluyendo las islas Galápagos (Keen 1971).

La concha del caracol púrpura es delgada de coloración gris-pardo y presenta ornamentaciones con nódulos agudos, presenta una espira apical reducida. La abertura pedal es grande con un labio externo delgado y manchas oscuras. La columela es lisa de color anaranjado con una mancha café anaranjada y violácea en los juveniles y en la mitad una mancha blanquecina en la pared parietal del labio interno. Presenta un canal sifonal corto (Figura 1). Se han registrado tallas de hasta 100mm y la talla promedio es de 64mm (Keen 1971)

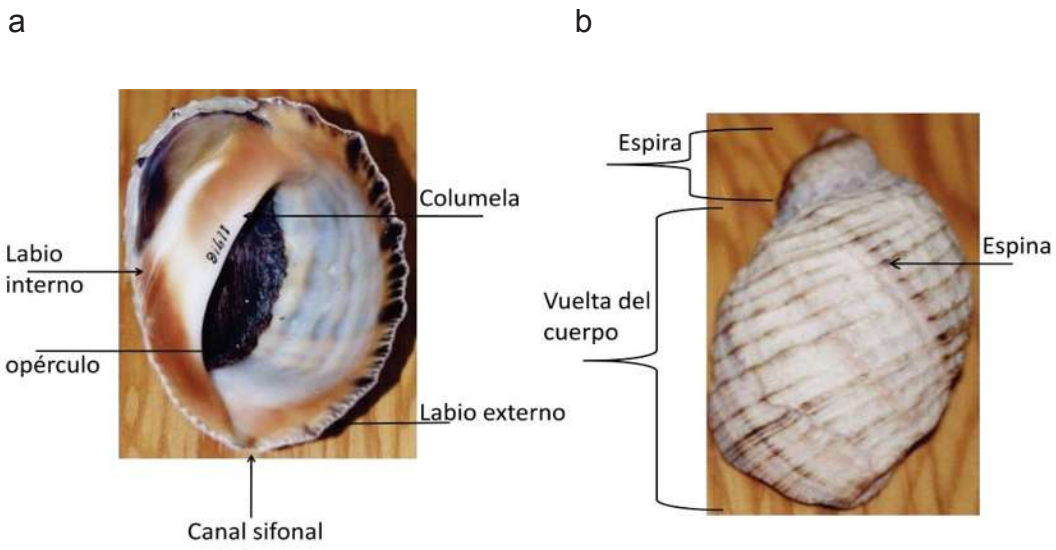

Figura 1 Esquema del caracol Púrpura *Plicopurpura pansa.* a) Vista de la abertura del cuerpo, b) vista dorsal.

El caracol púrpura ha sido objeto de estudios por su característica de producir un líquido incoloro en la glándula hipobranquial, que al ser expulsado es blanco y al contacto con el aire y la luz solar cambia a púrpura después de sufrir un proceso de oxidación. Existe una amplia cantidad de literatura concerniente a este colorante (Puchalska *et al*. 2004, Koren 2005, Koren 2006 y la bibliografía incluida en Cooksey 2001), el cual es producido por los caracoles de la familia Muricidae. Se sabe que este tinte ha sido utilizado desde el tiempo de los fenicios siendo la ciudad de Tiro (al sur de Líbano), en donde se concentraba el mercado de este colorante de ahí que se le conozca a este como púrpura de Tiro. Los primeros estudios se remontan a la era pre-química con una descripción del proceso de tinción por Plinio el viejo (Coocksey 2001). A mediados de 1800 Shunk aisla un compuesto de la especie *Purpura capillus*, a la cual llama puncina y posteriormente en 1880 extrae un colorante de una pieza de algodón teñido en Nicaragua con la especie *Purpura patula* y observa que es muy similar al obtenido anteriormente. Pero fue hasta 1909 cuando Friedlander determinó que el compuesto que se parecía al índigo era el 6,6'-dibromoíndigo, esto en *Murex* (*bolinus*) *brandaris* (Coocksey 2001) (Fig. 1 del capítulo 1).

Más recientemente el púrpura de Tiro ha seguido estudiándose hasta conocer su ruta metabólica. El precursor del tinte en el caracol es un líquido incoloro y se encuentra como sulfato de indoxil; éste al ser expulsado sale como indoxil, debido a la enzima sulfatasa que elimina el ion sulfato. Una vez fuera del organismo este sufre una oxidación foto-química para dar primero tirindoleninone el cual tiene la capacidad de producir dímeros que foto-oxidan y producen los cristales del compuesto final 6,6'-dibromoíndigo (Coocksey 2001, Benkendorff *et al*. 2001). También se ha intentado sintetizar el 6,6'-dibromoíndigo, logrando esto en el laboratorio a partir de diferentes compuestos como el 6-bromoindol (Tanoue *et al*. 2001) y 4-metil-anilina (Imming *et al*. 2001).

Benkendorff *et al.* (2000, 2001) encontraron algunos precursores del 6,6'-dibromoíndigo, como el tirindoleninona y el tiroverdín, en las cápsulas de huevos de los murícidos. Además encontraron que el tirindoleninona es el compuesto con mayor actividad antimicrobial, el tiroverdín, compuesto altamente bacteriostático y el 6-bromoisatín, un subproducto de la oxidación, presenta una actividad media como antimicrobial en las cápsulas de huevo de algunos murícidos. También se ha visto que el 6-bromoindirubin es un fuerte inhibidor del ciclo celular actuando sobre las proteínas GSK-3 (Meijer *et al*. 2003), por lo que puede ser un importante metabolito en estudios contra el cáncer.

Por otro lado se han realizado trabajos como los de Turok (1998) y Turok *et al*. (1998), donde abordan la historia y problemática del caracol púrpura como recurso para teñir. También se tienen estudios sobre su ciclo de vida, al estudiar el desarrollo larvario en condiciones del laboratorio. Después de haber sido puestos los huevos y al paso de un par de semanas, se presenta una primera larva que se desarrolla dentro del huevo y después de seis a ocho semanas de haberse presentado la primera fase larvaria, esta se transforma en una larva veliger de vida libre (Naegel 2004), es entonces cuando se rompen las cápsulas de los huevos para que estas larvas veliger pasen a formar parte del plancton en su estado natural.

El caracol púrpura presenta un lento crecimiento (Michel-Morfín *et al.* 2000, Ramírez-Rodríguez y Naegel 2003). Las diferencias de las densidades entre poblaciones están asociadas a las diferencias de los sustratos en las diferentes playas (García *et al*. 2004, Flores *et al*. 2007, García *et al*. 2007). La relación del tamaño del organismo y la cantidad de tinte que expulsan los organismos es otro tema que se ha estudiado. Se ha visto una correlación directa entre el tamaño del organismo y la cantidad de tinte. La periodicidad con la que se puede ordeñar los caracoles para evitar la mortalidad por ordeñas sucesivas es de 21 días (Michel-Morfín y Chávez 2000, Michel-Morfín *et al.* 2002, Chávez y Michel 2006).

La genética molecular ha ayudado a discernir la estructura genética y filogeográfica (Edwards y Beerli 2000, Wares y Cunningham 2001, Holland y Hadfield 2002), así como la historia evolutiva de diferentes especies (Collins *et al.* 1996, Marko y Vermeij 1999, Marko 1998).

La filogeografía es la subdisciplina de la biogeografía que trata con las relaciones históricas dentro y entre especies cercanas y se refiere a los principios y procesos que determinan la distribución geográfica de los linajes genealógicos (Avise 2000). Las estructuras filogeográficas entre especies que comparten el hábitat se pueden comparar para inferir procesos históricos (Avise 2000).

Del mismo modo se pueden realizar estudios comparativos de filogeografía, en donde deben existir especies que compartan distribución. El estudio comparativo más simple involucra linajes de varios pares de especies o poblaciones separadas por una barrera geográfica (Edwards y Beerli 2001). El reto de la filogeografía es utilizar información geográfica y de genealogías para inferir procesos históricos y demográficos que dieron forma a la evolución de los linajes.

La distribución actual de los organismos es producto de eventos geológicos que han ocurrido a lo largo de diferentes regiones y los cambios ambientales asociados. Dicha distribución puede ser explicada por fenómenos de vicarianza o eventos de colonizaciones repetidas. Uno de los casos de interés, es el levantamiento del Istmo de Panamá, que ocurrió hace aproximadamente 3.5-3.1

millones de años. Los estudios genéticos de especies hermanas encontradas una en el Pacífico oriental y la otra en el Atlántico occidental, han servido como ejemplo del papel que juega una barrera geográfica en la interrupción del flujo genético dando como resultado una especiación alopátrica (Marko y Jackson 2001, Marko 2002, Beu 2001). Además, estos casos proveen un útil e inusual sistema paleobiológico para un estudio evolutivo ya que el cese de intercambio de agua entre el Pacífico y el Atlántico impone un límite mínimo de tiempo en el que las especies hermanas han estado separadas. A pesar de que el levantamiento del istmo de Panamá está bien determinado por un dato geológico, los tiempos de divergencia de las especies hermanas actuales se asume que ocurrieron al final del levantamiento del istmo Panamá (Marko y Jackson 2001). Datos moleculares y paleontológicos recientes muestran que el aislamiento de las especies hermanas fue un evento gradual más que un evento abrupto (Coates *et al*. 1992, Collins *et al*. 1996).

Procesos geográficos históricos, como la división poblacional, rango de expansión y colonización a grandes distancias, se espera que produzcan distintos patrones en la distribución de los alelos y su relación entre ellos, por lo que se podría pensar que esos procesos pudieran ser inferidos a partir de los patrones de variación genética (Irwin 2002).

Para la conservación de especies es importante identificar los componentes de los linajes evolutivos para retener la máxima diversidad genética e incorporar información acerca de los procesos poblacionales históricos. Otra característica es comparar patrones dentro de las cuales, comunidades enteras han pasado por periodos substanciales de evolución independiente debido a eventos de vicarianza (Moritz y Faith 1998). Se ha propuesto que los análisis filogeográficos comparativos proveen una forma para investigar patrones de componentes genéticos de la biodiversidad como un elemento para determinar prioridades para la conservación o manejo de reservas ecológicas (Moritz y Faith 1998).

La filogeografía aplicada a la conservación ha permitido la identificación de unidades demográficas mínimas o poblaciones, que se comportan como unidades

reproductivamente independientes. El conocer la composición y distribución de las poblaciones permite un manejo más eficiente y científico de las poblaciones, ajustando las acciones en función de la abundancia y condición de cada una de las unidades de manejo identificadas.

El ADN mitocondrial (ADNmt) es útil y ampliamente utilizado para trabajar estos aspectos, ya que al ser heredado vía materna se pueden detectar los linajes matrilineales. El ADNmt no está sujeto a recombinación, estos linajes son jerárquicos y muestran clara relación entre individuos (Irwin 2002). Otra característica por la cual se utiliza este marcador es que el tamaño efectivo de la población para el ADNmt es de un cuarto del genoma nuclear (Riddle *et al*. 2000), lo cual permite que se pueda trabajar con números de muestras relativamente pequeños. Una de las regiones que se utilizan del ADNmt es el citocromo b el cual presenta una tasa de mutación hasta 10 veces más que el genoma nuclear y varía entre taxones por el orden de 5 veces (Avise 2004). El ADNmt tiene una tasa evolutiva relativamente alta y se hereda de manera uni-parental, por vía materna y esto ofrece un árbol génico no reticulado (bifurcado), y acumula variabilidad de modo rápido en ausencia de flujo génico, por lo que los estudios filogeográficos que utilizan marcadores moleculares, ofrecen una mayor resolución de los patrones intra-específicos de la variación geográfica que si se utilizan métodos no moleculares.

Actualmente no existen trabajos sobre filogeografía de las especies del género *Plicopurpura.* Los artículos en donde se citan alguna de las tres especies del género, son utilizados como un grupo externo para la filogenia del género *Nucella* (Collins *et al*. 1996), así como en la filogenia de artrópodos (Jeffrey *et al*. 1995), o como parte de la subfamilia Rapaninae en un estudio de filogenia y ecología histórica (Vermeij y Carlson 2000).

La falta de literatura relacionada con *P. pansa*, *P. columellaris* o *P. patula*, respecto a la estructura poblacional y filogeográfica, hace que sean especies de interés para su estudio. Así como las características que presenta el género *Plicopurpura* ya que presentan dos especies compartiendo su distribución, y la

tercera especie, habitante del Atlántico Occidental y que junto con *P. pansa* del Pacífico son un par de especies que pudieron presentar una especiación alopátrica y la posibilidad de que se haya presentado en evento de vicarianza. Además, dado que el caracol púrpura ha sido objeto de una explotación y puede ser una especie potencial a seguir explotándose hacen que un estudio filogeográfico comparativo sea un modelo de la estructura de las poblaciones en las diferentes regiones geográficas en las que se distribuye. Por esto, se pretende realizar un estudio de filogeografía del caracol purpura *Plicopurpura pansa* para sentar las bases que en un futuro inmediato ayuden a determinar áreas biogeográficas de interés para la conservación de la especie en cuestión y abrir la posibilidad de continuar con un estudio similar de las otras dos especies del género *Plicopurpura* y tratar de identificar los componentes de los linajes evolutivos para retener la máxima diversidad genética y tratar de incorporar información acerca de procesos poblacionales históricos.

II. Hipótesis

Existen diferentes metabolitos volátiles derivados de indol para cada una de las especies de *Plicopurpura pansa P*. *columellaris* y *P*. *patula*.

El levantamiento del istmo de Panamá permitió la dispersión de especies habitantes de la zona intermareal rocosa hacia el norte del Pacífico oriental. Dado el tiempo transcurrido desde el evento del levantamiento hasta esta época (3.5 ma aproximadamente), las poblaciones se pudieron establecer de tal modo que la estructura genética de las poblaciones del caracol púrpura *P. pansa,* a lo largo del Pacífico Mexicano está bien estructurada, permitiendo identificar diferentes grupos de poblaciones.

III. Objetivos

Conocer los metabolitos y sus derivados volátiles producidos por la glándula hipobranquial del caracol púrpura *Plicopurpura pansa* y compararlos con los presentes en *P. columellaris* y *P. patula*.

Analizar la estructura filogeográfica del caracol púrpura *Plicopurpura pansa* para conocer la relación de las poblaciones a lo largo de la costa del Pacífico Mexicano.

IV. Resultados

Capitulo 1

Brominated precursors of Tyrian purple (C.I. natural violet 1) from *Plicopurpura pansa* (Gould, 1853), *P. columellaris* (Lamarck, 1822) and *P. patula* (Linnaeus, 1758)

Felipe Javier López Chávez[ac], Patricia Ríos Chávez[a], Ken Oyama[b]

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Resumen

El colorante púrpura de Tiro y sus precursores han sido objeto de gran cantidad de estudios que involucran a varias especies de la familia Muricidae. Sin embargo no se tiene conocimiento previo de los precursores de los pigmentos de los caracoles *P. patula* y *P. columellaris,* especies pertenecientes a la familia Muricidae. Debido a las características del colorante 6,6'-dibromoindigo se tuvo que aplicar la técnica de derivatización seguida de la resonancia magnética nuclear para la especie de *P. pansa*. También se muestra el espectro de resonancia magnética nuclear del ^{1}H y ^{13}C del tirindolinona (6-bromo-2,2-bis-metilsulfanil-1,2-dihidro-3H-indol-3-ona), el cual es un aducto metano-tiol del tirindoleninona, que se presenta en *P. pansa*. La técnica de cromatografía de gases acoplada a masas se utilizó para detectar los productos indólicos bromados presentes en las especies de *P. pansa*, *P. columellaris* y *P. patula*. Los resultados obtenidos, publicados en la revista "Dyes and Pigments", muestran la presencia del tirindoleninone solo en la especie *P. pansa*, tanto en la glándula hipobranquial como en el exudado glandular. Por otro lado se encontró un compuesto presente en la glándula hipobranquial de *P. columellaris* y *P. patula* pero no en la de *P. pansa*, dicho compuesto es el 6-bromoindalín-2-ona (6-bromo-1,3-dihidro-2H-indol-2-ona).

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Brominated precursors of Tyrian purple (C.I. Natural Violet 1) from *Plicopurpura pansa*, *Plicopurpura columellaris* and *Plicopurpura patula*

Felipe Javier López Chávez [a,c,*], Patricia Ríos Chávez [a], Ken Oyama [b]

[a] Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Ciudad Universitaria, CP 58030 Morelia Mich, Mexico
[b] Centro de Investigaciones en Ecosistemas, Universidad Nacional Autónoma de México (UNAM), Ant. Carr. a Pátzcuaro 8701, Col. ex-Hda de San José de la Huerta, Campus Morelia, 58190 Michoacán, Mexico
[c] Departamento de Estudios para el Desarrollo Sustentable de Zonas Costeras, Universidad de Guadalajara, Gómez Farías 82, Colonia Centro, San Patricio-Melaque, Jalisco, Mexico

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ABSTRACT

Tyrian purple (C.I. Natural Violet 1) and its precursors have enjoyed much attention as various species of gastropods of the Muricidae family. However, few investigations have concerned the dye's precursors, namely *Plicopurpura columellaris* and *Plicopurpura patula*. Derivatization and NMR revealed that the purple pigment in *Plicopurpura pansa* is 6,6′-dibromoindigo. ^{1}H and ^{13}C NMR enabled tyrindolinone (6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3H-indol-3-one), a methanethiol adduct of tyrindoleninone from *P. pansa*, to be identified. GC/MS was used to identify the precursors of C.I. Natural Violet 1 from *P. pansa*, *P. columellaris* and *P. patula*. Tyrindoleninone (6-bromo-2-methylsulsulfanyl-3*H*-indol-3-one) was present in the milk and the hypobranchial gland of *P. pansa* only whereas an indole derivative, 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one), was present in the hypobranchial gland of *P. patula* and *P. columellaris* but not in *P. pansa*.

1. Introduction

Natural dyes extracted from animal and plant species have been used intensively in human activities throughout history. Of such dyes from animal origin, many are derived from the hypobranchial glands of several gastropods of the family Muricidae, the most famous being Tyrian purple (C.I. Natural Violet 1) [1,2] which is obtained from species of the genera *Murex*, *Bolinus*, *Purpura*, *Plicopurpura* and *Thais*. Depending on the species, the dye can be obtained in different purple and blue hues, due to the presence of brominated and non-brominated compounds [2]. *Plicopurpura pansa* is unique as it does not have to be sacrificed in order to obtain the dye in so far as it can be "milked" periodically [3–5].

In 1880, Schunck investigated the purple dye obtained from *P. pansa* and showed that it was like indigo, but not identical to it. In 1909, Friedlander identified the purple product from the Mediterranean *Murex (Bolinus) brandaris* as 6,6′-dibromoindigo [2]. In *P. pansa* the major component of the purple dye was identified by HPLC as 6,6′-dibromoindigo **5** (90%), along with 6-bromoindigo (9%), and 6,6′-dibromoindirubin (1%) by Withnall et al. [6].

The tyrindoleninone **3** (red) and tyrindolinone **6** (yellow) intermediates in the production of the purple dye were identified, with TLC with 6-bromoisatin **7** as another product from different muricid species [Baker and Duke, 1971 in 2; 7; Hiyoshi and Fujise 1992 in 2; 8; 9]. LC/MS has been used to identify Tyrian purple precursors such as tyrindoxyl sulfate **1** and tyrindoxyl **2** in *Dicathais orbita* [10].

GC/MS has been useful in the identification of 6,6′-dibromoindigo **5** precursors as well as other brominated compounds in some muricids; Benkendorff et al. [11,12] found several known and unknown brominated compounds.

Nuclear magnetic resonance (NMR) has been used to characterize the intermediates precursors of natural and synthetic dyes. Tyriverdin **4** structure, the former precursor of 6,6′-dibromoindigo **5** was obtained by chemical synthesis [13]. On the other hand 6,6′-dibromoindigo **5** has been synthesised from different compounds such as: 6-bromoindole and 4-methyl-aniline [14,15]. The metabolic

* Corresponding author. Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Ciudad Universitaria, CP 58030 Morelia Mich, México.
E-mail address: jlopez@costera.melaque.udg.mx (F.J. López Chávez).

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path of 6,6′ dibromoindigo **5** is already known. The organism produces a sulfatase (purpurase), that eliminates sulfate ion to give tyrindoxyl **2**, when exposed to oxygen it changes to tyriverdin **4** which, in the presence of sunlight, gives the permanent form 6,6′-dibromoindigo **5** (Fig. 1) [8].

The purpose of the "milk" in gastropods is not yet fully understood, speculations includes its role in feeding where it may be secreted to anaesthetize prey, or as a stimulant to facilitate detachment of the gastropod from its surroundings. It may be used in reproduction, since the secretory activity, in some muricids, increases during breeding season [Fretter and Graham, 1994 in 16]; there are indole derivatives in egg masses and there appears to be an influence of genital ducts on indigoid biosynthesis during the reproductive season [16]. Indole derivatives of 6,6′ dibromoindigo **5** have been found in different muricids and it has been demonstrated that these derivatives can have antimicrobial activity [11,12]. It has been reported that 6 bromoindirubin is a strong GSK inhibitor [17]. Several investigations on the hypobranchial glands of the Muricidae family have revealed that the number and nature of precursors involved in the production of Tyrian purple differs among species [12]. Consequently, it is possible that different precursors could be found in the species of *Plicopurpura*.

The genus *Plicopurpura* has only three species, *P. pansa*, *Plicopurpura columellaris* and *Plicopurpura patula*, the former two live in the Pacific and *P. patula* lives in the Atlantic; these three species are predators of the intertidal rocky shore and they also produce Tyrian purple and expel it, but only *P. pansa* yields enough "milk" to use it to dye. *P. pansa* is the species that the Mixtecan people from Oaxaca, Mexico use to dye the cotton to make their clothes, and handcrafts. The Tyrian purple and its precursors have been studied in *P. pansa* but not in *P. columellaris* and *P. patula*. In this study, we characterized the 6,6′-dibromoindigo **5** and the

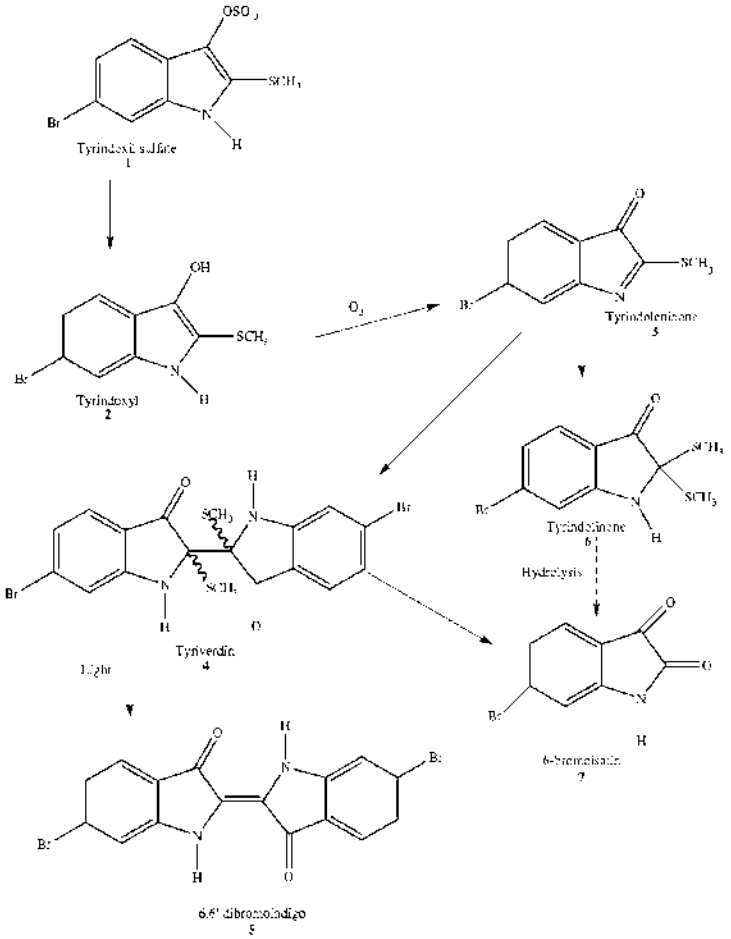

Fig. 1. The generation of the 6,6′-dibromoindigo (taken from Gaskey 2001 [2]).

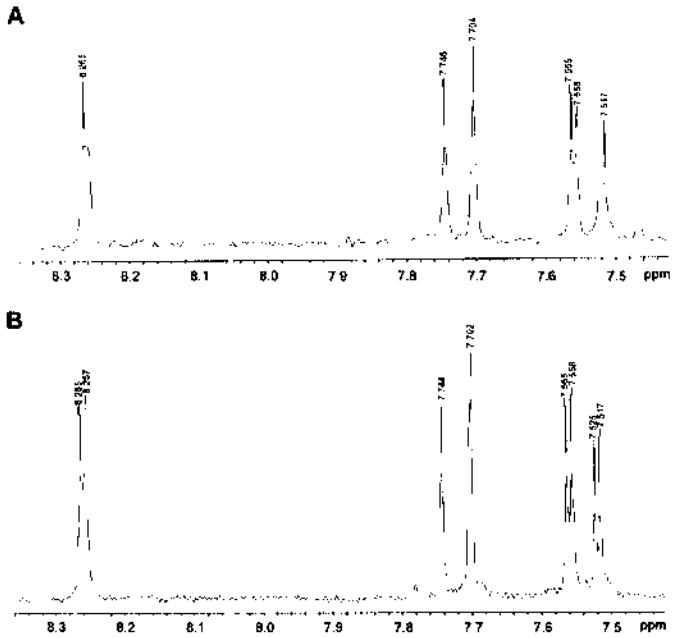

Fig. 2. ^{1}H NMR spectrum of synthetic and natural 6,6′-dibromoindigo isolated from milk of *P. pansa* (200 MHz, $CDCl_3$ and TMS as internal standard).

precursors along with another volatile indole derivatives on *P. pansa* from the milk and hypobranchial gland in order to use them to characterize the 6,6′-dibromoindigo **5**, the precursors and the indole derivatives from *P. columellaris* and *P. patula*.

2. Experimental

2.1. Extraction of natural dye

50 ml of the glandular secretion ("milk") was obtained from a total of 150 organisms of *P. pansa* at Peñitas, Michoacán, Mexico. It was obtained stimulating the operculum, pressing softly with the thumb, and collecting the "milk" into a dark bottle in order to avoid photochemical oxidation. A portion of the "milk" collected was exposed 8 days to sunlight to obtain 6,6′-dibromoindigo **5** crystals. Crystals were refluxed in ethanol for 2 h and a second reflux with ethyl benzoate to obtain pure purple crystals [18].

Hypobranchial glands from fresh *P. pansa* (8 organisms from Peñitas, Michoacán), and frozen *P. columellaris* (15 organisms from Tenacatita, Jalisco) and frozen *P. patula* (14 organisms from Piedra Escondida, Quintana Roo, all beaches from Mexico) were dissected out from snails and macerated with methanol–chloroform (1:1 v/v), the next steps were the same as in the "milk" extract. The identification of brominated compounds was accomplished using GC/MS.

2.2. Chemical synthesis

The chemical synthesis of 6,6′-dibromoindigo **5** was synthesised according to the procedure of Tanoue et al. [14].

2.3. N-N′-Bis(trifluoroacetyl) derivatives

The purple crystals were refluxed with 2 ml of chloroform and 2 ml trifluoroacetic anhydride for 4 h until the initial violet solution became orange. Complete evaporation gave a brown solid, which was dissolved in $CDCl_3$ and the proton NMR spectra were recorded immediately using a Varian model Mercury Plus at 400 MHz, with tetramethylsilane as internal standard.

2.4. Isolation of precursors

The "milk" collected from the snails was soaked in methanol/chloroform (1:1 v/v), 12 h, then decanted and the solvent replaced.

Table 1
Volatile brominated compounds in extracts from the hypobranchial gland of *P. pansa*, *P. columellaris* and *P. patula*.

Compound	Rt	Major fragments	*P. pansa* (milk)	*P. pansa*	*P. columellaris*	*P. patula*
6-Bromo-2-methoxy-3*H*-indole-3-one **8**	7.43	MI 239, 241 mf 222, 224, 168, 170	+	+	+	+
6-Bromoindalin-2-one **9**	10.31	MI 211, 213			+	+
Tyrindoleninone **3**	11.25	MI 255, 257 mf 240, 242, 212, 214, 182, 184, 133, 75	+	+		
6-Bromo-2-methylsulfinyl-3*H*-indole-3-one	13.84	MI 271, 273, mf 224, 226, 168, 170	+			
6-Bromoisatin **7**	14.11	MI 225, 227 mf 177, 179, 170, 172	+			

Table 2
Unidentified brominated indoles from the glandular extract (milk) of *P. pansa*, and hypobranchial gland of *P. pansa*, *P. columellaris* and *P. patula*.

Molecular Ion	Rt	Major fragments	*P. pansa* (milk)	*P. pansa*	*P. columellaris*	*P. patula*
229, 231	7.44	197, 199, 170, 172, 28		+	+	+
239, 241	7.57	229, 231, 197, 199, 170, 172	+			
253, 255	8.70	224, 226, 168, 170	+			
271, 273	8.81	256, 258, 224, 226, 208, 210	+			
255, 257	9.82	224, 226, 168, 170	+			
223, 225,	10.98	211, 213, 182, 184			+	
255, 257	11.03	240, 242, 227, 211, 213, 182, 184, 171, 101				+
257, 259	11.08	229, 231, 211, 213, 197, 199, 182, 184			+	+
255, 257	11.93	245, 247, 240, 242, 198, 200, 170, 172	+			
255, 257	12.12	239, 241, 211, 213, 182, 184	+			
317, 319, 321[a]	12.44	302, 304, 306, 224, 226, 168, 170	+			
271, 273	13.84	224, 226, 168, 170	+			
225, 227	14.12	197, 199, 170, 172	+			
237, 239	14.64	208, 210, 44, 32, 28			+	+
255, 257, 259[a]	16.10	240, 242, 244, 210, 212, 182, 184			+	+
299, 301	18.89	257, 259, 242, 244	+			
289, 291	21.23	261, 263, 154	+			

[a] Dibromo compound.

This was repeated three times with a last soak being overnight. The extracts were then mixed and evaporated to dryness in a rotary evaporator and resuspended in chloroform (5 ml). The chloroform extract was separated by column chromatography using a silica gel column (Sigma 70–230 mesh, 4 × 60 cm). The column was eluted with chloroform. The fractions were further separated on preparative TLC plates (Silica Gel 60) using dichloromethane/hexane (1:1 v/v).

2.5. Analysis of precursors

The structure of the isolated precursor was determined by GC/MS (Hewlett Packard GC mod. 5972, with a HP5MS (crosslinked 5% PH ME Siloxane)) 30 m × 0.25 mm × 0.25 μm film thickness, coupled to a low-resolution mass analyzer (Hewlett Packard mod. 5972 MSD). The operating conditions were: initial temperature 150 °C for 3 min and then increased at a rate of 4 °C/min to a final temperature of 300 °C which was maintained for 20 min. Helium was used as the carrier gas with a constant flow of 1 ml/min; one microliter of sample was injected with a split ratio of 50:1. The electron beam energy in the mass spectrometer was 70 eV and the source temperature was 230 °C.

3. Results and discussion

Chemical synthesis of 6,6′-dibromoindigo **5** gave 47% yield, slightly lower than that reported by Tanoue et al. [14]. The synthesis of 6,6′-dibromoindigo **5** was used to confirm the natural dye, this compound is insoluble in any solvent at ambient temperature, but it has been found that it is conveniently converted to the *N,N′*-bis(trifluoroacetyl) derivative by a treatment with trifluoroacetic anhydride [18].

The *N,N′*-bis(trifluoroacetyl) derivatives from synthetic and natural dye obtained from "milk" collected from *P. pansa* snails, were chemically indistinguishable. Both derivatives showed the three proton multiplets in the ^{1}H NMR spectrum δ 8.26, d, J = 1.3 Hz, 1H, Ar**H**; δ 7.72, d, J = 8.3 Hz, 1H Ar**H**; δ 7.54, dd, J = 8.3,1.5 Hz, 1H, Ar**H** (Fig. 2). We did not obtain the ^{1}H NMR of the dye in the hypobranchial glands of *P. columellaris* and *P. patula* due to the low concentration of crystals of 6,6′-dibromoindigo **5**.

The analysis of glandular secretion ("milk"), obtained from *P. pansa*, by column chromatography revealed seven colored compounds and one insoluble red compound, which did not pass through the silica column as Benkendorff et al. [12] found in frozen

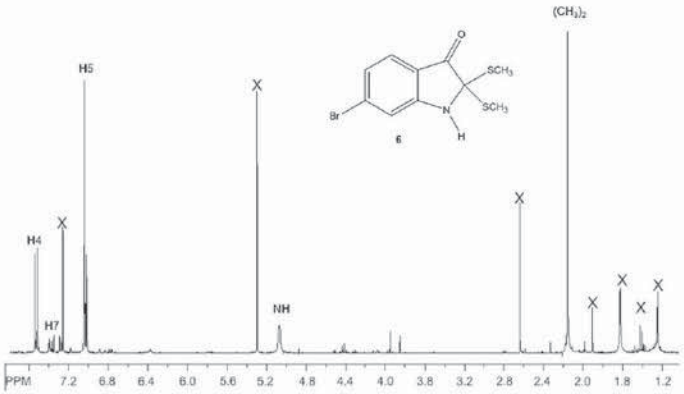

Fig. 3. ^{1}H NMR spectrum of 6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one (tyrindolinone) isolated from milk of *P. pansa* (400 MHz, $CDCl_3$ and TMS as internal standard).

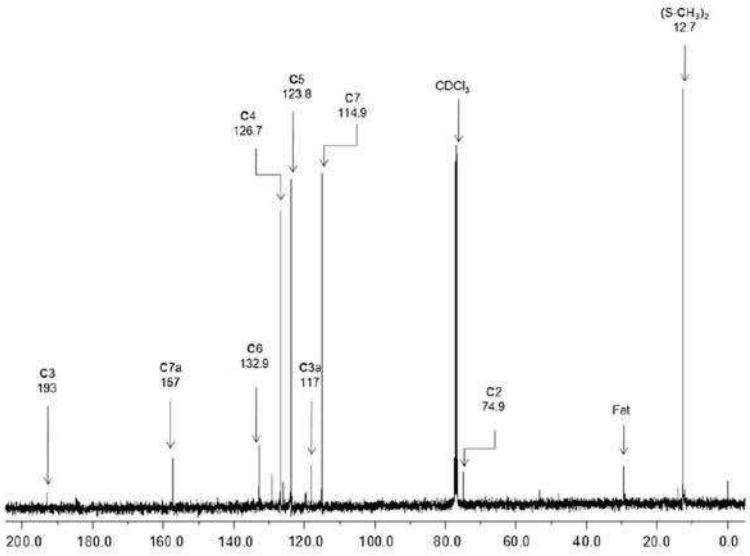

Fig. 4. ^{13}C NMR spectrum of 6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one (tyrindolinone) isolated from milk of *P. pansa* (400 MHz, $CDCl_3$ and TMS as internal standard).

eggs of *D. orbita* and *Trunculariopsis trunculus*. The components from the second and fourth bands were successfully isolated from a chloroform extract by thin-layer chromatography using dichloromethane and hexane (1:1, v/v). The second band broke down on silica to produce five bands: the orange component was identified as tyrindoleninone **3** by GC/MS, which was present only in *P. pansa* (Table 1). Benkendorff et al. [12] found that egg capsules of all muricids contain tyrindoleninone **3**, which is a metabolite that has antimicrobial activity.

GC/MS analysis of the "milk" from *P. pansa* revealed four compounds that could be identified as tyrindoleninone **3**, 6-bromoisatin **7**, 6-bromo-2-methylsulfinyl-3*H*-indole-3-one and 6-bromo-2-methoxy-3*H*-indole-3-one **8** (Table 1); the last two were identified by comparison with the MS data given by Benkendorff et al. [12]. There were also eleven brominated compounds detected in trace amounts, which were determined as brominated in base of the mass spectra pattern (Table 2). Two dibromo indole compounds were distinguished due to the distinctive triplet in the mass spectra present in the dibromo compounds (Table 2). Tyrindoleninone (6-bromo-2-methylsulfanyl-3*H*-indol-3-one) **3** was the most abundant compound with a retention time of 11.25 min; and M^+ *m*/*z* 255, 257, (^{79}Br, ^{81}Br) major fragments 242, 240,133 and 75. When the sample of the isolated tyrindoleninone **3** was analyzed by NMR spectroscopy, two weeks after the initial GC/MS analysis, the tyrindoleninone **3** had changed to tyrindolinone (6-bromo-2,2-bis-methylsulfanyl-1,2-dihydro-3*H*-indol-3-one) **6**, the ^1H NMR demonstrated the presence of tyrindolinone **6**, showing a singlet at δ 2.168 corresponding to the $(SC\mathbf{H}_3)_2$, protons (6H), with additional peaks at δ 7.53, d, $J = 8.3$ Hz, 1H Ar**H**; δ 7.019, d, $J = 8.3$ Hz, 1H Ar**H**; δ 7.05, m, 1H Ar**H**; δ 5.08, brs, 1H, N**H** (Fig. 3). The carbons in the ^{13}**C** NMR spectrum of tyrindolinone **6** resonated at 400 MHz, the signals were: δ 12.738 ($\mathbf{C}H_3$)^2, δ 74.96 (**C**-2), δ 114.897 (**C**-7), δ 117.968 (**C**-3a), δ 123.784 (**C**-5), δ 126.741 (**C**-4), δ 132.913 (**C**-6), δ 157.276 (**C**-7a) and δ 193.065 (**C**-3) (Fig. 4). These data confirm the

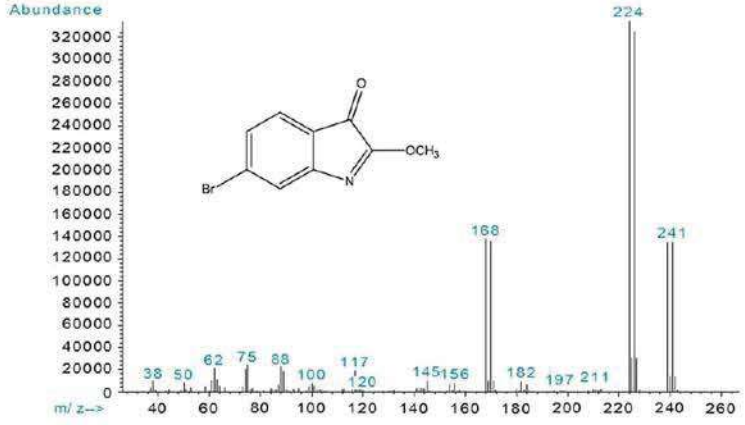

Fig. 5. Mass spectrum of 6-bromo-2-methoxy-3*H*-indole-3-one.

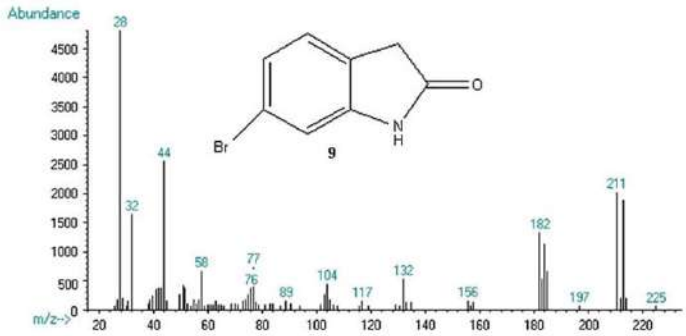

Fig. 6. Mass spectrum of 6-bromo-1,3-dihydro-2*H*-indole-2-one (6-bromoindalin-2-one).

presence of 10 C's which correspond to tyrindolinone **6**, while tyrindoleninone **3** has 9 C's.

6-Bromoisatin (6-bromo-1*H*-indole-2,3-dione) **7** was found in the milk of *P. pansa* in small amounts and identified by GC/MS with a retention time of 14.11 min and M^+ *m/z* 225, 227 (^{79}Br, ^{81}Br); major fragment ions were observed at *m/z* 197, 170 and 63. Benkendorff et al. [11] proposed that 6-bromoisatin **7** is a by-product of the decomposition of tyriverdin **4**. However we found tyrindoleninone **3** as a major compound and this finding may support the idea of Cooksey and Withnall [8], who proposed that 6-bromoisatin **7** may be a product of the hydrolysis of tyrindolinone **6** (dashed arrow in Fig. 1). None of the hypobranchial extracts of *P. pansa*, *P. columellaris* and *P. patula*, or the "milk" of *P. pansa*, showed peaks at *m/z* 418, 420, 422 which are characteristic of tyriverdin **4**. We concluded that the absence of this compound could be the result of the low solubility of tyriverdin **4** in all solvents.

Another compound found in the "milk" of *P. pansa* and in the hypobranchial glands of the three species, was 6-bromo-2-methoxy-3*H*-indol-3-one **8** (Table 2) which had a retention time of 7.43 min and a M^+ *m/z* 239, 241, major fragments 224, 226, 168,170 (Fig. 5). This compound was reported by Benkendorff et al. [12] in egg masses of *D. orbita*, *Ceratostoma erinaceum* and *T. trunculus* and it was suggested that 6-bromo-2-methoxy-3*H*-indol-3-one **8** could be a by-product due to the use of methanol as solvent.

On the other hand, in the hypobranchial gland of *P. columellaris* and *P. patula* an indole derivative was present, the mass spectra library matched to 5-bromoindalin-2-one (5-bromo-1,3-dihydro-2*H*-indole-2-one), but as in 5-bromoisatin, this is more likely to be 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one) **9**, since most, if not all, the compounds have the bromine atom in sixth position. Benkendorff et al. [12] reported 6-bromoindoxyl (6-bromo-1,2-dihydro-3*H*-indole-3-one) in egg masses of some muricids, and M^+ *m/z* 211,213, with major fragments 102, 104. Our compound, with a retention time of 10.31 min and M^+ *m/z* 211, 213, with major fragments 182, 184 (M–CO), and a small fragment 156, 158 (M–CH_2NH) which increases the *m/z* 28 fragment (Fig. 6), confirm that the compound was the 6-bromoindalin-2-one (6-bromo-1,3-dihydro-2*H*-indole-2-one) **9**. This compound was found as a result of the pyrolysis of 6,6′-dibromoindirubin in Tyrian purple obtained from *Murex trunculus* [19].

We detected other unidentified volatile brominated compounds in the extracts of hypobranchial glands from *P. columellaris* and *P. patula* but not in *P. pansa* by comparing the mass spectra with the ones reported by Benkendorff et al. [12] (Table 2).

Secondary metabolites differ from primary metabolites in having a restricted distribution in the animal and plant kingdoms. They can be found in only one species or a taxonomically related group of a species, whereas the basic primary metabolites are found throughout the kingdoms [20]. Their direct role in plant metabolism is not yet well documented. However, their ecological role, herbivore interaction, and chemotaxonomy have been well established [21]. In the last 30 years, nearly 10,000 different natural products have been isolated from marine organisms. These compounds have eventually served as leads for the development of modern nucleoside drugs for antiviral chemotherapy [17,22].

Chemotaxonomy has contributed to species classification when the organisms have morphological similarities, as had been shown in lichens [23], fungus [24], and marine species [25]. Comparing the secondary profile of a species, it is possible to observe a group characterized by the presence of one compound and another group by the presence of one type of metabolite could be different. So the presence of a kind of metabolite could be used, in addition to morphological data, to define species. *P. pansa* had been considered as the same species as *P. columellaris* [26], and as a subspecies of *P. patula* [27], afterwards, studying the morphology of the radulae the three species were reported as different species [28]. The results presented in this paper support the idea proposed by Castillo [28].

4. Conclusion

The chemical nature of the precursors and brominated indoles of the purple dye in the hypobranchial gland and the extracts of the glandular secretion from *P. pansa*, were identified according to their known fragmentation patterns. The tyrindoleninone **3** was a major compound in *P. pansa*. On the other hand, the presence of 6-bromoindaline-2-one **9** was detected in the hypobranchial gland of *P. columellaris* and *P. patula* but not in *P. pansa*.

According to the results presented here, the three species had different brominated indole derivatives supporting the morphological [28] and molecular data [29] that distinguish the three species.

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Capitulo 2

Phylogeography of the purple snail *Plicopurpura pansa* (Gould 1893) along the Mexican Pacific shore.

Felipe J. López-Chávez[1, 2] Omar Chassin-Noria[2] Patricia Ríos-Chávez[2], Víctor Rocha[3] and Ken Oyama[3]

Resumen

En el presente trabajo se estudió la estructura genética del caracol púrpura *Plicopurpura pansa* a lo largo del Pacífico mexicano para determinar su estructura filogeográfica. Se colectaron 211 organismos de 17 localidades del Pacífico mexicano y una de Costa Rica. Se obtuvieron 100 haplotipos del gen del citocromo b del ADN mitocondrial. La estructura genética se analizó con un AMOVA, obtuvimos una red de haplotipos con el algoritmo de "Median Joining" y un análisis de distribución "mismatch" bajo el supuesto de poblaciones en expansión. Los niveles de diversidad genética fueron uniformes presentando en general una diversidad haplotípica alta y una diversidad nucleotídica baja. Las diferencias genéticas entre las poblaciones fueron bajas pero significativas en un par de ellas, son las dos poblaciones que se encuentran más al sur de la zona de muestreo. La red de haplotipos y la distribución "mismatch" indican poblaciones en expansión, tal vez recuperándose de un cuello de botella. Algunas especies de gasterópodos habitantes de la zona intermareal rocosa presentan baja o nula diferenciación genética en distancias similares a las aquí presentadas y los resultados obtenidos son similares presentando una escasa diferenciación entre poblaciones y poca estructura filogeográfica. Las poblaciones mexicanas de P. pansa se recuperan de un cuello de botella causado posiblemente por la última glaciación.

Abstract

Aim Purple snail *Plicopurpura pansa* inhabits the rocky intertidal zone along the tropical Eastern Pacific. In the present we analyzed the genetic structure of *P. pansa* to elucidate its phylogeographic structure along the Mexican Pacific.

Location Along the Mexican Pacific.

Methods 211 organisms were collected from 17 localities including one beach from Costa Rica. We obtained 100 haplotypes from the cytochrome b gen of mitochondrial DNA. Genetic structure was analyzed via standard AMOVA. A haplotype network was built using the Median Joining algorithm and last a mismatch distribution analysis was performed under the hypothesis of expanding populations.

Results Genetic differences among populations were low but significant in two of them from all the others, these two populations were the southernmost ones collected. Haplotype network and mistmatch distribution analyses showed that the Mexican populations are expanding, or recovering from a bottleneck.

Main conclusion Some species of rocky intertidal shore gastropods presents low or no genetic differentiation among populations in distances similar to those presented in the present work, and the results obtained here are similar, since the genetic differentiation among Mexican populations is low as well as the phylogeographic structure.

Keywords

Cytochrome b, mtDNA, population genetics, Muricidae, rocky intertidal shore, Tropical Eastern Pacific.

INTRODUCTION

Marine intertidal gastropods phylogeographic structure seems to be related to larval dispersal, some of these snails show starlike politomies in their haplotype network, the genetic structure shows panmixia and there is little or no phylogeographic structure (Lee and Boulding 2007, Ayre et *al*. 2009, Lee and Boulding 2009, Crandall *et al*. 2008)

Patterns of migration of planktonic larva can determine the genetic structure of marine species along their geographic distribution (Avise 2004). Larva movement along shore has been studied in diverse groups of organisms as crabs (Shanks 1983), in fishes (Neethling *et al*. 2008), in asteroids (Yasuda *et al*. 2009) and in gastropods (Vargas *et al*. 2004) indicating that marine currents can connect populations separated as much as 100 km away. The larva may not always be passively dispersed, some larva can swim and move horizontal and vertical and migrate from the surface currents to the inner currents that are generally slower than the upper ones, however some species have different larval behavior, and this along with the physical mechanisms makes larval transport difficult to measure (Pineda et al. 2007). Besides biological factors, physical conditions as internal tides, bores and eddies may affect the genetic structure (Pineda 1999, Pineda *et al*. 2007, Avise 2004).

Plicopurpura pansa has a planktonic larva and the time it spends in plankton is not well known, Chávez and Michel (2006) made an estimate from the time eggs are laid to the moment the first recruits appear, and it is about four months. Studies that deal with genetic structure of rocky intertidal gastropods have shown little or no differentiation among populations (Van de Broeck *et al*. 2008, Crandall *et al*. 2008). Glaciations are an important factor that determines the phylogeographical pattern in many marine and terrestrial species (Hewitt 1996, 2004, Crandall *et al*. 2008) by appearing and disappearing habitats as sea level rises or lowers resulting in a change in genetic structures. These changes associated to glacial maximum cycles and the presence of a planktonic larva may play an important role in shaping the phylogeographical pattern of the *P. pansa*.

The purple snail *P. pansa* suffered an intense exploitation in the early 80's, which resulted in a drastic decrease in population densities and loss of organisms bigger than 60mm (Turok 1998). This may lead to a loss of genetic variability of southern Mexican Pacific populations, due to the loss of a part of the gene pool Regard the abovementioned we used cytochrome b gene to provide an estimate of the population structure in the Mexican Pacific shore populations of *P. pansa* and a fine scale phylogeography in order to elucidate some demographic events.

MATERIALS and METHODS

Sampling

A total of 221 organisms of *Plicopurpura pansa* were collected in 2006 from different locations at Mexican Pacific shore (table 1): Pescadero and Cabo San Lucas in Baja California Sur, Mazatlán in Sinaloa, Platanitos and Isla Maria Madre in Nayarit, Clarion island from Colima, Faro de Bucerias, San Juan de Alima and Peñitas in Michoacán, Barra de Potosí, Playa Ventura and Punta Maldonado in Guerrero, Puerto Angel, Carrizalillo and Huatulco in Oaxaca all beaches in Mexico, and in Punta Catedral in Costa Rica. Samples were preserved in 95% ethanol until genetic analysis.

DNA extraction, PCR and sequencing analysis.

Individual genomic DNA was extracted from 200mg of foot tissue following the phenol-chloroform-alcohol method (Palumbi, 1996). We amplified a 716bp fragment of the cytochrome b gene using primers 14,841 and 15,573 (Anderson *et al*. 1981 in Collins *et al*. 1996). PCR amplification was performed in a 25 µL volumes containing: 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.36 µM of each primer, 1 x buffer (10 x buffer contains 500 mM KCl and 100 mM tris HCl (pH 8.3)), 1 unit Taq, 1 x BSA, 50 to 200 ng DNA template. Thermo cycling conditions were: initial denaturation 1 min at 95° C, followed by 35 cycles of 1 min at 95º C, 1 min at 50º C and 1 min at 72º C, then a final extension of 5 min at 72° C. PCR products were run in 0.8% agarose gel visualized with ethidium-bromide. PCR products were cleaned with QIAquick Gel Extraction Kit (QIAGEN).

Sequencing reaction was performed for both forward and reverse strands using Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The products were run in a 3100-Avant™ (Applied Biosystems) automated DNA sequencer. Complementary strands were proofread and aligned in Sequencher 4.7.

Sequence alignment

The aligning of sequences was completed with Sequencher 4.7.

Population genetics

The nucleotide diversity (π) and haplotype diversity (h) were estimated with DnaSP (Rozas *et al.* 2003) and Arlequin 3.11 (Excoffier *et al*. 2005) respectively.

The geographical relationship among populations, taking into consideration the difference of haplotypes, was quantified using a molecular variance (AMOVA, Excoffier *et al*. 1992) analysis carried out with Arlequin 3.11(Excoffier *et al*. 2005). The AMOVA uses the genetic distances among haplotypes along with the frequencies.

We calculated pairwise Fst values among populations (taking into consideration each sampling site as a population) the analysis used 10,000 replicates to test significance of values.

Phylogeographic structure

In order to see the haplotypes evolution, haplotype frecuency and geographical representation a haplotype network was constructed using maximum parsimony and the median joining algorithm (Bandelt *et al.* 1999).

Fu´s Fs test was performed for possible population expansion. The distribution of observed and expected nucleotide site pairwise difference with the constant population model in order to obtain an estimate of theta initial and final was calculated as well as Tau, parameters that are necessary to get the Population growth-decline model implemented in DnaSP (Rozas *et al*. 2003). The Harpending´s Raggedness index was calculated to test for the goodness of fit of the data in the mismatch analysis.

RESULTS

Genetic diversity

We sequenced a 716 bp fragment of mitochondrial cytochrome b gene from 221 individual *P. pansa* from 17 localities distributed along 3000 km (Figure 1). We found 100 haplotypes which are place on a map to see haplotype frequency distribution by population (Figure 2). The percent averages of C, T, A and G, were 17.59%, 39.93%, 24.70% and 17.79%, respectively. The variation of C, T, A and G content among populations was no more than 0.16% and the only nucleotide that varied was T. in the 716 bp fragment we recognized 96 polimorphic sites, from which 40 sites were parsimony informative sites and 56 singleton variable sites.

The haplotype diversity (h) ranged from 0.5 to 0.9 (Table 1), it appears to be high if we take into consideration that it ranges from 0 to 1 (Grant and Bowen 1998). The haplotype diversity of populations north of Banderas bay seems to be a little bit lower than the populations south of Banderas bay, with the exception of Puerto Angel that has a h=0.6 a little bit lower than the rest of these south populations. Nucleotide diversity (π) is low ranging from 0.0009 to 0.006 (Table1) considering 0 for no divergence and above 10% for deep divergence (Grant and Bowen 1998).

Population structure

The AMOVA showed that the global Φst result across all samples was 0.022 (P 0.045) is low but significant, this is so because when examining Fst among all pairs of populations Huatulco and Costa Rica are different from 12 out 16 populations (Table 2). After that we analyzed Huatulco and Costa Rica as a separate group but the difference among groups was not significant (data not shown).

The gene flow, presented in table 2 as Nm, shows high numbers of individuals capable of reproduce migrating among all populations, here again Huatulco and Costa Rica presents the lowest numbers of Nm between them and

the rest of populations. Here there is another population that has relatively low transport of individuals per generation among populations that is Clarion Island (Table 2).

Phylogeographic structure

Median-joining tree exhibits two major star-like polytomies with central high frequency haplotypes (haplotype Pp1 n=25, and Pp6 n=43) separated by three bp differences (Figure 3), the haplotypes near the dotted line in figure 3 have descendant haplotypes for both clades

Fu's Fs test for all sites shows negative results and significant (Table 1) as would be expected for populations that have undergone recent demographic changes. The mismatch distribution analysis showed a unimodal representation of results (Figure 4), hence a fit to the sudden expansion model concordance with the expansion population model raggedness index r=0.0128.

Discussion

The high haplotype diversity (h) is common among rocky intertidal mollusks (Table 3). In *Nerita* species (*N. albicilla* 529 samples in 60 localities and *N. plicata* 653 in 73 localities). Crandall *et al*. (2008) found high haplotype doversity (0.94 to 0.99) in a 658-bp region of the mitochondrial cytochrome oxidase subunit I gene. In *Tectarius striatus*, another rocky intertidal snail, the mean haplotipic diversity was 0.875 (n = 109 from 11 islands), sequencing a 482bp and a 511bp fragments of cytochrome b and cytochrome oxidase I, respectively (Van den Broeck *et al*. 2008). *Litorina breviculata* from Korean waters presents a low h (Table 3) (Kim *et al*. 2003). Here in P pansa we found values of haplotype diversity ranging from 0.615 to 1. On the other hand, the nucleotide diversity (π) was low, ranging from 0.004 to 0.0062. The genetic diversity seen in *P. pansa* is similar to the rocky intertidal species (Table 3). Avise (2000) and Grant and Bowen (1998) concluded that populations with high haplotype diversity and low nucleotide diversity can be considered recent stable growing populations, this as a result of expanding populations via founder effect or recovering from a bottleneck.

The populations from Platanitos to the north presents an h and π not different from the south of Banderas Bay populations, but these northern populations have less haplotypes than the southern populations (Figure 2 and 3), this may not represent difference on genetic variability (see below the AMOVA results) but, when making an analysis of variance just taking into consideration the number of haplotypes, grouping northern of Banderas bay and southern of Banderas bay populations, the media are statistically different this done with JMP software (data not shown). The difference on haplotype number among northern and southern populations may be related to the last glacial maximum (LGM), where the sea level lowered about 120m (Rohling *et al*. 1998) and the coast also changed worldwide, but in the region where the southern populations did not changed much, according to the map presented by Hewitt (2000), this may tell us that the northern populations may have disappeared and reestablished after LGM resulting in loss haplotypes due to the loss of habitat and this would be the cause of cyclical local

extinction of demes followed by recolonization with the sea level rise (Crandall *et al*. 2008).

While two groups can be seen with the number of haplotypes, no structure is shown by the AMOVA. First we calculated the AMOVA with all populations as one group and showed little difference but significant been the difference in Huatulco and Costa Rica populations, the difference of the later population may be an artifact of sampling, since we only have three samples of that population and Huatulco is the southernmost Mexican population sampled and the difference small but significant may be related to larval dispersion due to the movements of the currents, since there is an eddie in Tehuantepec gulf (López-Calderón et al. 2006) that can act as a barrier from the rest of populations by keeping larvae close to the hatching beach. After that we separated into two groups being the north group Pescadero, Cabo San Lucas, Clarión, Mazatlán, and Platanitos and the rest of populations the south group, corresponding this separation with the difference in haplotype number that separates these two groups. The data from pairwise Fst and Gene flow, given by Nm, confirms what AMOVA shows, the lack of genetic structure that is due to the high gene flow among all Mexican populations but Huatulco (Table 2). These high values of gene flow are related to the presence of the free living larva in P. *pansa* which can spend up to four months as plankton. The difference of Huatulco from all the other populations may be due to the presences of an eddie in the gulf of Tehuantepec, it is present during winter and may be present for several months (Trasviña *et al*. 1995, Filonov and Trasviña 2000, López-Calderón *et al*. 2006), it is an anticyclonic gyre so the forces of the eddie may maintain the larvae from going northward and it may take the larva more than a 1000km west offshore (López-Calderón *et al*. 2006). Costa Rica is the other population that presents low genetic flow and the presence of an eddie in the gulf of Papagayo and the gulf of Tehuantepec eddie (López-Calderón *et al*. 2006) represents barriers to gene flow in and that can be the reason of having low genetic gene flow with the rest of populations sampled.

The low genetic differentiation and high gene flow is found in some intertidal snails that present a planktonic larva (Ellingson and Krug 2006 0.08; Lee and Boulding 2009 0.05, 0.01 and 0.05; Hurtado *et al*. 2007 0.07, 0.04; Kim *et al*. 2003 0; Van den Broeck *et al*. 2008 0.02; Crandall *et al*. 2008 0.011, 0) and bivalve *Placopecten magellanicus* with planktonic larvae 0.005 (Kenchington *et al*. 2006). The results presented here (Tables 1 and 2) are not so different from the ones presented in the above mentioned papers.

The haplotype network shows two common haplotypes, and from them derives smaller polytomies that define two clades, one in the north and the other in the south (Figure 3). The former includes Pescadero, Cabo San Lucas, Clarion, Mazatlán and Platanitos. The south clade includes localities from Tenacatita in Jalisco to Huatulco in Oaxaca. The haplotype number for each population ranges from 2 to 19. The south group presents higher diversity as we can see in table 1, where the northern populations have less haplotypes than the south populations. During LGM sea level modified the costal line from Banderas Bay to the north, the continental shelf in this part of the Mexican Pacific is about 80Km and about 200m deep around Maria Islands nowadays (Torres-Orozco *et al*. 2005), and from Tehuantepec gulf to the south being pretty similar as it is today due to the continental shelf, this may be the reason why the northern group, that includes Platanitos, Mazatlán, Pescader, Cabo San Lucas and Maria islands. Many temperate species have been found to have a distribution pattern related to the LGM in both the northern and in the southern hemispheres (Hewitt 2000, 2004). Another hypothesis may be, that the species is recovering from an overharvesting of the tyrian purple dye produced by the purple snails *P*. *pansa,* situation that was given by the Japanese “Imperial Purple” company, they were the responsible of the decrese of population’s numbers in the early 1980’s (Turok *et al*. 1998), situation that lead the species to a bottleneck and is recovering.

The starlike conformation of the haplotype network represents a growing population, and it is often related to panmictic populations (Avise 2000). This situation is shown with the data obtained herein, since population differentiation is

low and the gene flow, analyzed in Arlequin as Nm is, in most of comparisons, high being more than one (Table 2). This two evidence show that the populations presented here are expanding populations. All the species in table 3 that presents a high h and low π presents a star-like haplotype network all related to recent grow population or recovering from a bottleneck.

The third analysis performed was the mismatch distribution, the results of Fu's Fs show populations of recent creation or recovering from a bottleneck and would be concordant with the starlike politomy and the high haplotype diversity and low nucleotide diversity. This analysis shows that our data adjust to the expected under the growing population assumption (Fig. 3).

The three analyses presented herein, trying to elucidate the phylogeographic pattern of *Plicopurpura pansa*, give enough information to think that the Mexican Pacific populations are in expansion process recovering from a bottleneck. While the difference in haplotype number would suggest the presence of two groups and that would support the hypothesis of glaciations, there is not enough genetic data to support that idea, the number of unique hapltypes differs from populations north of Banderas bay and those present in the south part of the Mexican Pacific (Figures 2 and 3). Otherwise the lost in genetic variability may be the result of the over exploitation that suffered *P. pansa* in the early 1980's (Turok *et al*. 1998).

Locality	n	Number haplotypes	h	π	Fu's Fs
Pescadero	15	8	0.533	0.004	-16.85 p 0.0
Cabo Sn Lucas	14	9	0.643	0.0035	-15.95 p 0.0
Clarión	5	5	1	0.003	-2.86 p 0.01
Isla Maria	7	6	0.857	0.005	-3.86 p 0.004
Mazatlán	13	9	0.692	0.0041	-12.99 p 0.0
Platanitos	14	9	0.642	0.0049	-13.30 p 0.0
Tenacatita	9	7	0.777	0.0048	-6.32 p 0.001
Faro de Bucerias	13	11	0.846	0.0055	-10.88 p 0.0
Peñitas	15	11	0.733	0.0047	-15.28 p 0.0
San Juan de Alima	16	14	0.875	0.0058	-15.03 p 0.0
Barra de Potosi	17	14	0.823	0.0053	-17.43 p 0.0
Playa Ventura	17	13	0.764	0.0053	-17.51 p 0.0
Punta Maldonado	19	18	0.947	0.0062	-19.11 p 0.0
Puerto Angel	14	8	0.615	0.0044	-14.07 p 0.0
Carrizalillo	15	13	0.866	0.0055	-13.93 p 0.0
Huatulco	15	11	0.733	0.0051	-14.48 p 0.0
Costa Rica	3	2	0.666	0.0009	-2.19 p 0.0
Mean		9.88	0.765	0.0045	-12.47 p 0.00088

Table 1. Diversity indices of *P. pansa* along the Pacific coast.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		0.070	0.095	0.077	-0.044	0.418	-0.025	0.139	0.072	0.056	0.039	0.062	0.014	0.083	0.005	0.080	0.528
2	6.69		-0.020	-0.038	-0.024	0.165	0.026	-0.007	-0.026	-0.035	-0.027	-0.032	-0.032	-0.029	-0.020	0.073	0.308
3	4.75	Inf		-0.048	-0.021	0.117	0.046	-0.017	-0.022	-0.039	-0.032	-0.025	-0.016	-0.019	-0.005	0.105	0.248
4	6.03	Inf	Inf		-0.028	0.154	0.027	-0.014	-0.031	-0.034	-0.042	-0.035	-0.029	-0.025	-0.026	0.081	0.302
5	Inf	Inf	Inf	inf		0.246	-0.064	0.008	-0.017	-0.031	-0.037	-0.026	-0.057	-0.001	-0.042	0.046	0.363
6	0.697	2.53	3.76	2.76	1.53		0.318	0.019	0.068	0.140	0.141	0.071	0.160	0.146	0.193	0.303	0.157
7	Inf	18	10	17	Inf	1.1		0.093	0.039	0.026	0.010	0.025	-0.019	0.029	-0.023	-0.002	0.428
8	3.10	Inf	Inf	Inf	62	26	4.9		-0.031	-0.010	0.000	-0.034	0.006	-0.005	0.020	0.130	0.124
9	6.46	Inf	Inf	Inf	Inf	6.88	12	inf		-0.030	-0.021	-0.034	-0.025	-0.034	-0.012	0.084	0.191
10	8.42	Inf	Inf	Inf	Inf	3.07	18	Inf	inf		-0.036	-0.024	-0.029	-0.022	-0.016	0.088	0.271
11	12	Inf	Inf	Inf	Inf	3.03	51	2002	inf	inf		-0.021	-0.031	-0.024	-0.023	0.074	0.234
12	7.62	Inf	Inf	Inf	Inf	6.52	19	Inf	inf	inf	inf		-0.023	-0.033	-0.014	0.071	0.153
13	35	Inf	Inf	Inf	Inf	2.62	Inf	77	inf	inf	inf	inf		-0.029	-0.034	0.056	0.258
14	5.52	Inf	Inf	Inf	Inf	2.93	16	Inf	inf	inf	inf	inf	inf		-0.012	0.073	0.285
15	98	Inf	Inf	Inf	Inf	2.09	Inf	24	inf	inf	inf	inf	inf	inf		0.036	0.298
16	5.71	6.30	4.26	5.64	10	1.15	Inf	3.34	5.43	5.20	6.26	6.52	8.50	6.34	13.46		0.390
17	0.447	1.13	1.52	1.15	0.879	2.69	0.668	3.53	2.12	1.34	1.63	2.76	1.44	1.25	1.18	0.780	

Table 2. Fst pairwise difference above diagonal and gene flow given by Nm below diagonal, inf infinite. 1 Cabo San Lucas, 2 Pescadero, 3 Platanitos, 4 Mazatlán, 5 Isla María, 6 Clarión, 7 Tenacatita, 8 Faro de Bucerías, 9 Sn Juan de Alima, 10 Peñitas, 11 Barra de Potosí, 12 Punta Maldonado, 13 Playa Ventura, 14 Puerto Ángel, 15 Carrizalillo, 16 Huatulco and 17 Costa Rica.

Sp	h	π	Marker	Reference
Nerita albicilla	0.98	0.021	COI	Crandall *et al.* 2008
N. plicata	0.99	0.013	COI	Crandall *et al.* 2008
P. pansa	0.76	0.004	Cyt b	Present work
Tectarius striatus	0.87	n/a	Cyt b/COI	Van den Broeck *et al.* 2008
N. scabricosta	0.91	0.005	COI	Hurtado *et al.* 2007
N. funiculata	0.99	0.008	COI	Hurtado *et al.* 2007
Littorina keenae	0.81	0.003	ND6/Cyt b	Lee and Boulding 2007
L. breviculata	0.30	-	Cyt b	Kim *et al.* 2003
L. breviculata	0.29	-	ND6	Kim *et al.* 2003
Batillaria cumingi	0.85	0.012	COI	Kojima *et al.* 2004
L. sitkana	0.10	0.0003	Cyt b	Lee and Boulding 2009
L. subrotundata	0.30	0.001	Cyt b	Lee and Boulding 2009
L. scutulata	0.39	0.003	Cyt b	Lee and Boulding 2009
L. plena	0.77	0.006	Cyt b	Lee and Boulding 2009
Cellana tramoserica	0.71	0.001	COI	Ayre *et al.* 2009
Bembicium nanum	0.92	0.006	COI	Ayre *et al.* 2009
Morula marginalba	0.86	0.004	COI	Ayre *et al.* 2009
Haustrum vinosa	0.83	-	COI	Ayre *et al.* 2009
Alderia modesta	0.98	0.021	COI	Ellingson and Krug 2006

Table 3. Comparison of haplotype (h) and nucleotide (π) diversity of some rocky intertidal mollusks.

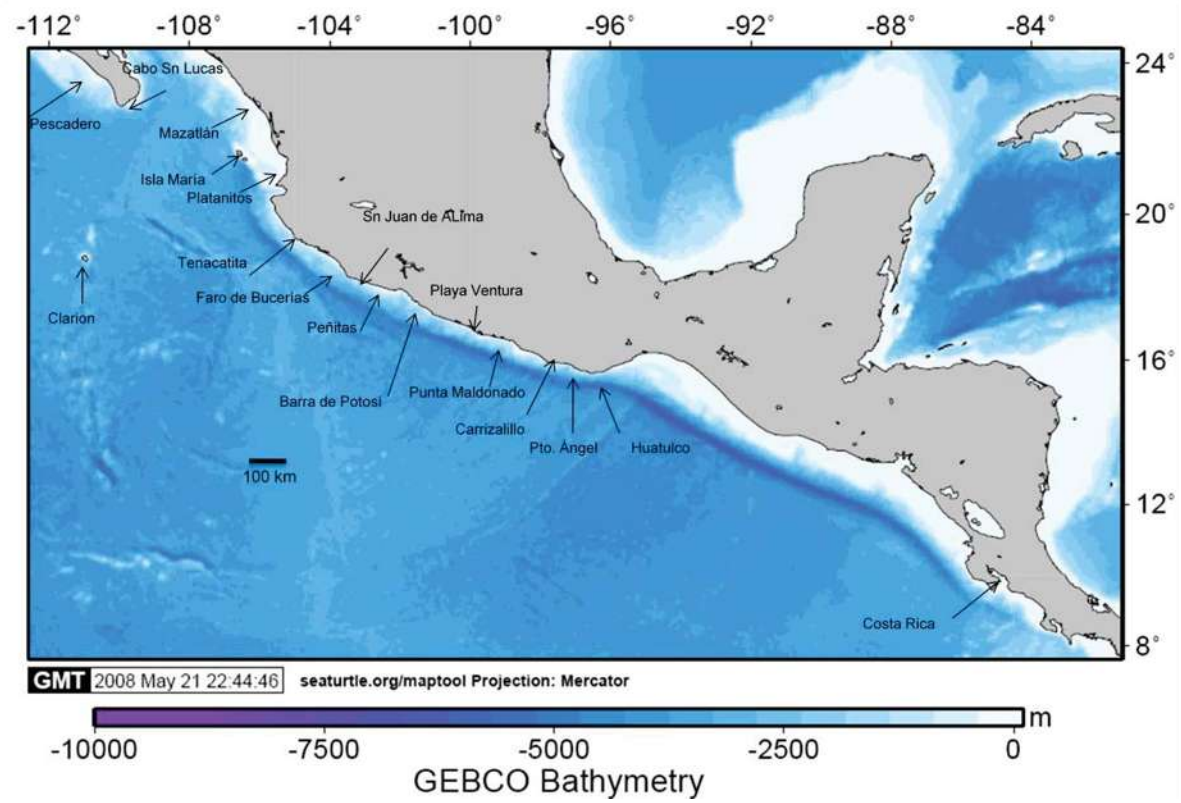

Figure 1. Sample sites from the Mexican Pacific and Costa Rica.

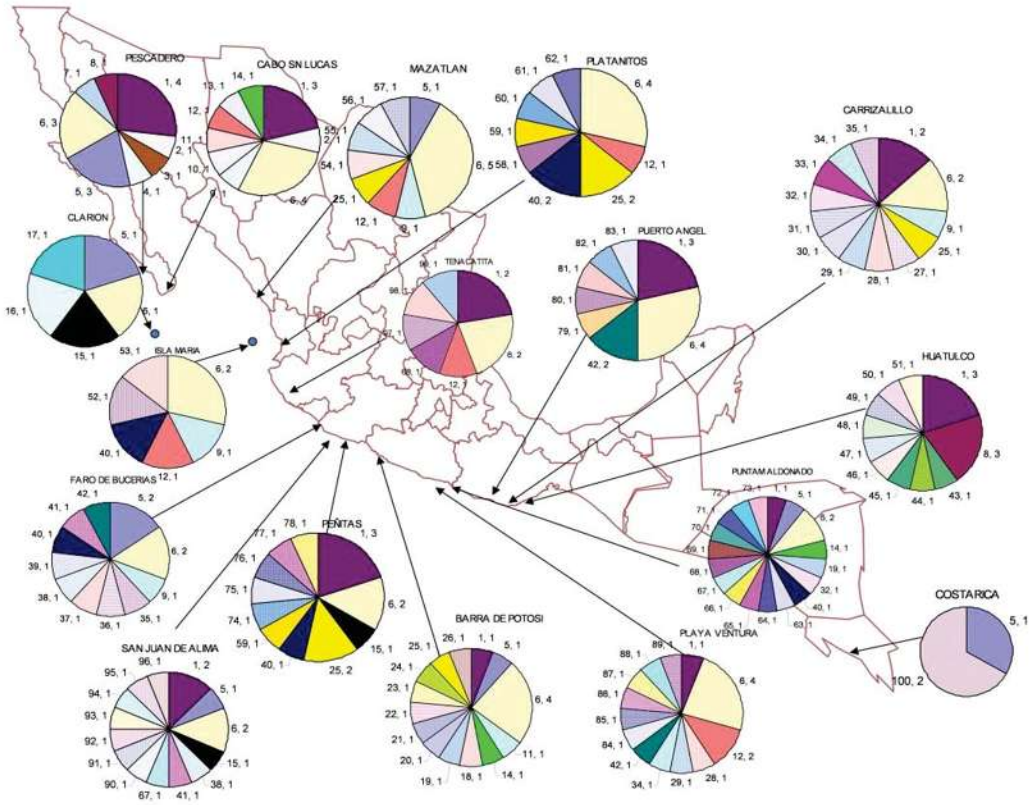

Figure 2. Distribution of haplotypes along the pacific shore of *P. pansa*.

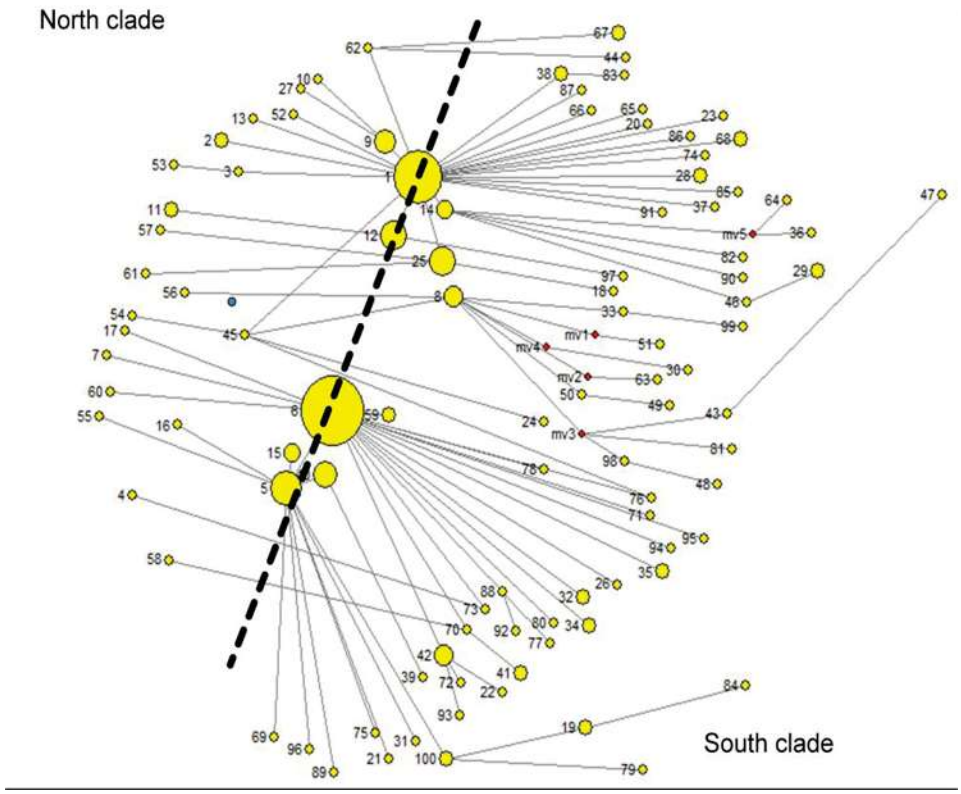

Figure 3. Haplotype network of *P. pansa*.

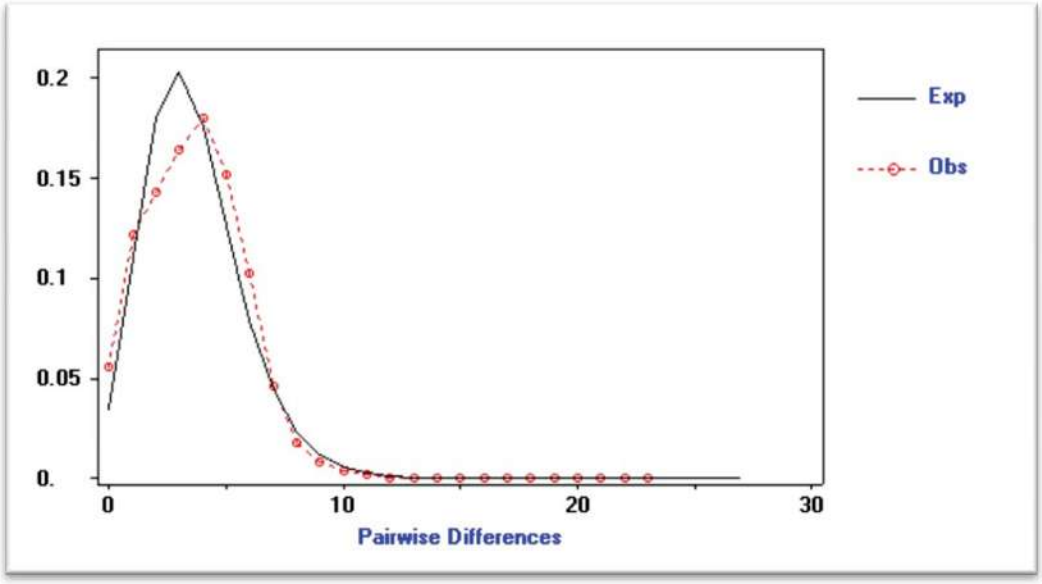

Figure 4. The mistmach distribution graph with a population growth scenario.

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V Discusión General

La taxonomía del género *Plicopurpura* ha sido objeto de cambios. Las tres especies que comprende este género fueron consideradas dentro del género *Purpura* (Keen 1971). Posteriormente, Kool (1988) propuso ubicar estas tres especies en un género aparte, dado que considera que existe evidencia suficiente y propone retomar el género *Plicopurpura* propuesto por Cossmann (1903). Por otro lado *Plicopurpura pansa* ha pasado de ser considerada una sola especie junto con *P. columellaris* (Wellington y Curis 1983), tomando en cuenta la morfología de la concha, a una subespecie de *P. patula* (Kool 1988; Skoglund 1992), y a una especie separada *Plicopurpura pansa* (Castillo 1992), en base a una comparación del tracto digestivo y de la rádula.

Dado que se ha tenido que llegar a diferenciar a estas especies con respecto de su morfología interna, podría ser de utilidad el buscar otras herramientas que nos ayuden en la separación de especies, para lo cual se cuenta actualmente con técnicas moleculares capaces de resolver los detalles que no muestran los resultados morfológicos. Por otro lado existen los metabolitos secundarios producidos por los organismos vivientes los cuales pueden ser, en algunas ocasiones, exclusivos para cada una de las especies o para un grupo de especies, esto se ha encontrado ya en organismos marinos como en esponjas (Thiel *et al.* 2002) y en gorgónidos (Gerhart 1983), la quimiotaxonomía se ha utilizado para diversos organismos. Con respecto de las especies *P. pansa*, *P. columellaris* y *P. patula* los metabolitos encontrados en ellas nos indican diferencias entre las tres por lo que estos datos estarían apoyando el trabajo de Castillo (1992). Por otro lado los marcadores moleculares, en un análisis preliminar, nos muestran, al igual que los metabolitos, una diferencia entre las tres especies. Lo anterior se basa en un análisis filogenético con el algoritmo de el vecino más cercano realizado con el programa Mega 4.1 (Tamura *et al*. 2007) y este separa a las tres especies con una inclusión de un organismo de *P*. *columellaris* en el grupo de *P*. *pansa* (Figura 1), esto puede ser ocasionado por la hibridación, situación que se presenta entre *P*. *pansa* y *P*. *columellaris*, y lo que

pudo haber sucedido es que en algún momento hibridaron un macho de *P*. *pansa* y una hembra de *P*. *columellaris* y con el tiempo se eliminó cualquier rasgo fenotípico de *P*. *columellaris* pero se conserva las mitocondrias de la madre, situación que se ha corroborado en algunos organismos (Wares y Cunningham 2001). Por lo que tanto los resultados de los metabolitos secundarios derivados del indol de la glándula hipobranquial y los resultados preliminares de las secuencias del gen del citocromo b apoyan el trabajo de Castillo (1992), en el sentido de que son tres especies diferentes *P*. *pansa*, *P. columellaris* y *P*. *patula*.

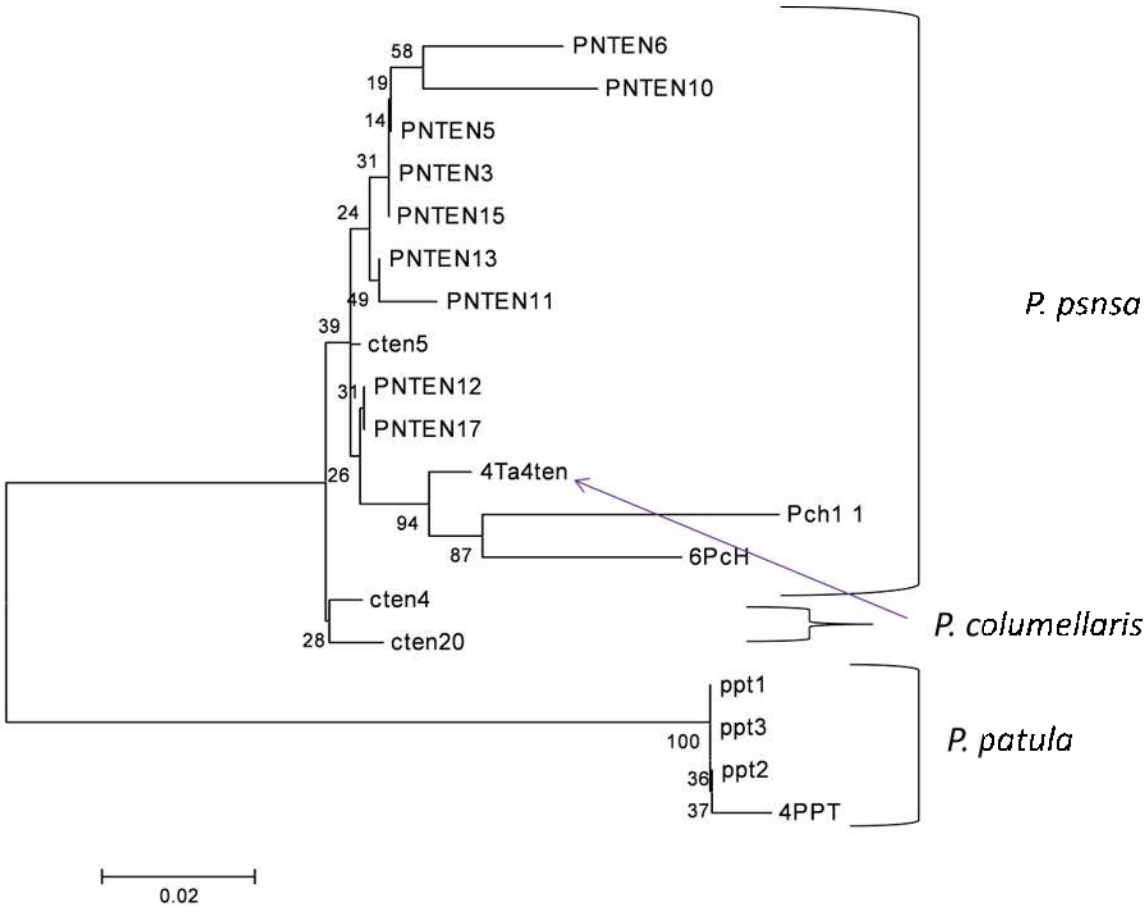

Figura 1. Árbol construido con el algoritmo del vecino más cercano *P*. *pansa*, *P*. *patula* y *P*. *columellaris*.

VI Perspectivas

El estudio de los metabolitos secundarios como una herramienta en la sistemática se ha utilizado en algunos grupos de animales marinos como, esponjas y corales entre otros. Al encontrar diferentes metabolitos derivados del indol en cada una de las tres especies de Plicopurpura, sugiere que la quimiotaxonomía se podría explorar con más detalle en futuros trabajos de moluscos gasterópodos. Por otro lado el que algunos precursores o derivados de indol tengan propiedades de antibiótico o bacteriostáticos así como inhibidores del ciclo celular podríamos sugerir el buscar otros compuestos presentes en el tinte del caracol *P. pansa* y probar sus posibles propiedades ya sea bactericidas, bacteriostáticas o como inhibidores del ciclo celular, específicamente en la investigación del cáncer.

Por otro lado el no encontrar estructura poblacional ni filogeográfica, a lo largo de la costa del Pacífico Mexicano con la ayuda de ADN mitocondrial, es probable que si se utiliza un marcador que tenga una tasa de mutación más alta, tal vez encontremos la variabilidad suficiente como para detectar estructura y tal vez comprobar la estructura entre el grupo que marcamos como del norte, de Nayarit hacia el norte de México y de Jalisco hacia el sur de México, que si bien se puede observar pequeñas diferencias entre la red de haplotipos pero no son estadísticamente significativas, se puede utilizar esta nominación de grupos para utilizarla con marcadores como los microsatélites que si bien son nucleares, ya se tiene un primer intento con el marcador mitocondrial, por lo que se propone desarrollar microsatélites para las tres especies de *Plicopurpura*.

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Glosario

Derivatización- Reacción química utilizada para modificar un compuesto químico en uno similar con la finalidad de cambiar las propiedades del compuesto original, al compuesto obtenido se le nombra “derivado”. Tales cambios pueden ser en su reactividad, solubilidad, punto de ebullición o de fusión. Estas nuevas características se utilizan para cuantificar o separar el derivado.

NMR- Resonancia Magnética Nuclear es una característica que presentan los núcleos magnéticos en presencia de un campo magnético al cual se le aplica un pulso electromagnético que ocasiona que el núcleo absorba energía del pulso electromagnético y radie esta energía de regreso, la cual se puede medir.

GC/MS- Cromatografía de gases acoplada a espectrometría de masas. Es una técnica que combina las propiedades de la separación de compuestos volátiles (cromatografía de gases) y la identificación de los compuestos de acuerdo a su patrón de fragmentación (espectrometría de masas).

TLC- Cromatografía de capa fina. Es una técnica que se utiliza para separar mezclas, esto con la ayuda de placas (de vidrio, poliuretano o aluminio) que contienen una capa de material adsorbente, generalmente sílica gel. Esta es conocida como la fase estacionaria. Esta placa se carga con la muestra y se coloca en una cámara con solvente o una mezcla de estos, que actúa como la fase móvil.

δ- Símbolo del desplazamiento químico que es la diferencia (en partes por millón), entre la frecuencia de resonancia del protón que se analiza y el protón del tetrametilsilano (TMS) que es el estándar que se utiliza en el análisis de NMR.

M+ - Masa de la molécula original, llamado ion molecular.

m/z- Relación carga a masa, m es la masa del ion y z su carga en unidades de carga electrónica.

AMOVA- Análisis de varianza molecular es un análisis estadístico para conocer la variación molecular entre poblaciones de una especie.

Diversidad haplotípica (h)- La probabilidad de que dos haplotipos escogidos al azar, en una muestra, sean diferentes.

Diversidad nucleotídica (π)- Medida de variación genética y se define como el número promedio de diferencias de nucleótidos por sitio entre dos secuencias, escogidas al azar, de una población.

Distribución "mistmach"- La distribución del número observado de diferencias entre pares de haplotipos. La distribución es multimodal para poblaciones en equilibrio y unimodal para poblaciones en expansión.

Nm- Número de migrantes, capaces de reproducirse, entre dos poblaciones por generación.