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revbiolmar@gmail.com

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ARTICLE

# Calibrating the chitons (Mollusca: Polyplacophora) molecular clock with the mitochondrial DNA cytochrome C oxidase I gene

Calibrando un reloj molecular para quitones (Mollusca: Polyplacophora)  
utilizando el gen mitocondrial que codifica para el citocromo oxidasa I

Cedar I. García-Ríos<sup>1</sup>, Nivette M. Pérez-Pérez<sup>1</sup>,  
Joanne Fernández-López<sup>1</sup> and Francisco A. Fuentes<sup>1</sup>

<sup>1</sup>Departamento de Biología, Universidad de Puerto Rico en Humacao, Avenida José E. Aguiar Aramburu, Carretera 908 km 1,2, Humacao, Puerto Rico. cedar.uprh@gmail.com

**Resumen.**- Especies hermanas separadas por el Istmo de Panamá se han utilizado para estimar tasas de evolución molecular. Un reloj molecular calibrado con un evento geológico bien estudiado puede ser de utilidad para proponer hipótesis sobre la historia evolutiva. El Istmo de Panamá emergió completamente hace 3,1 millones de años (m.a.), pero se ha evidenciado que comunidades asociadas a costas rocosas y arrecifes quedaron aisladas en fechas más tempranas (5,1-10,1 m.a.). Se determinó la divergencia de segmentos del gen mitocondrial citocromo oxidasa I (COI) para 2 pares de especies hermanas de quitones. El par *Stenoplax purpurascens* del Caribe y *S. limaciformis* del Pacífico oriental tropical, son especies muy similares fenotípicamente. Las especies del segundo par, *Acanthochitona rhodea* del sur del Caribe y *A. ferreirai* de las costas pacíficas de Panamá y Costa Rica, fueron tratadas como la misma especie hasta la reciente descripción de *A. ferreirai*. Utilizando ejemplares recolectados en ambas costas de Costa Rica se compararon secuencias homólogas del gen COI. Para el par de especies del género *Stenoplax* se determinó una divergencia total de 11,4% pares de bases (bp). Para el par de especies del género *Acanthochitona*, se encontró una divergencia de 10,4-10,5% bp. Estos valores de divergencia del COI permiten calcular razones de divergencia de 3,7% / m.a. (aislación hace 3,1 m.a.) hasta 1,1% / m.a. (aislación temprana). Esta es la primera calibración del gen COI como reloj molecular para los polioplacóforos.

**Palabras clave:** Istmo de Panamá, reloj molecular, COI, *Stenoplax*, *Acanthochitona*

**Abstract.**- Sister species separated by the Isthmus of Panama have been used to estimate the rate of molecular evolution. A molecular clock calibrated with a well studied geological event can be useful to propose evolutionary history hypotheses. The Isthmus of Panama emerged completely 3.1 million years ago (mya), but fossil evidence has shown that rocky shore and reef mollusk communities were isolated early (5.1-10.1 mya). The divergence of segments of the mitochondrial gene cytochrome oxidase I (COI) was determined for 2 pairs of sister species of chitons. The pair *Stenoplax purpurascens* of the Caribbean and *S. limaciformis* of the tropical Eastern Pacific consists of phenotypically similar species. The species of the second pair, *Acanthochitona rhodea* from the south of the Caribbean and *A. ferreirai* of the Panama and Costa Rica Pacific coast, were treated as the same species until the recent description of *A. ferreirai*. Homologous fragments of the COI gene were compared using specimens collected on both coasts of Costa Rica. For the *Stenoplax* genus pair, a total divergence of 11.4% base pairs (bp) was determined. The *Acanthochitona* pair showed a divergence of 10.4-10.5% bp. These values allow calculated divergence rates from 3.7% / mya (3.1 mya isolation) to 1.1% / mya (early isolation). This is the first calibration of the COI as a molecular clock for the polyplacophora.

**Key words:** Isthmus of Panama, molecular clock, COI, *Stenoplax*, *Acanthochitona*

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## INTRODUCTION

Molecular clocks are useful for determining the time during which phylogenetic events occur. A molecular divergence analysis, used to calibrate a molecular clock, requires the knowledge of 2 facts: first, the time at which species is genetically isolated to give rise to 2 sister species and secondly, the certainty that the sister species

used for the calibration were isolated at that time, and not previously, through other speciation processes.

The Isthmus of Panama is a recent barrier that separated multiple populations of marine organisms, in what is today the Caribbean and tropical eastern Pacific (Knowlton & Weigt 1998). It was assumed that the last moment of

genetic exchange between populations of eastern tropical Pacific and Caribbean populations corresponds with the onset of the final closure of the Isthmus of Panama, which geological evidence situate at 3.1 million years ago (mya). The assumption that the sister species were isolated at the time when the exchange of seawater stopped completely (3.1 mya), was the hypothesis used by many researchers (Bermingham & Lessios 1993, Schubart *et al.* 1998, Hellberg & Vacquier 1999, McCartney *et al.* 2000, Haye *et al.* 2002, Collin 2003, Lessios *et al.* 2003, Morrison *et al.* 2004, Williams & Reid 2004, Duda & Kohn 2005, Duda & Rolán 2005). However, other researchers propose dates prior to this one (Cronin & Dowsett 1996, Knowlton & Weigt 1998, Marko 2002) arguing that because the lifting of the isthmus was gradual and the early stages of the water flow constriction could be barriers for some taxa, among them the deep-water fishes and the invertebrates intolerant to silt in their respiratory or filter structures (Coates & Obando 1996, Collins 1996). Several researchers provide evidence suggesting that the sister species associated with reef habitat diverged earlier, as compared to the mangrove habitat species (Knowlton *et al.* 1993, Knowlton & Weigt 1998, Lessios 2008, Miura *et al.* 2010). Considering gastropods (*Tegula* geminate pair) and bivalves (arcid) with fossil record for the calibration, Marko (2002) suggests that the divergence time for sister pairs of mollusks associated with rocky shore occurred between 5.1 to 10.1 mya.

The cytochrome oxidase I (COI) gene, can be used as a molecular chronometer, which is an appropriate tool to estimate the time at which gene flow was interrupted between 2 populations, given that the vicariant event occurred within a few million years (Knowlton & Weigt 1998). Nuclear protein coding gene Histone H3 suffers substitutions less frequently, so it's not appropriate for the analysis of divergence for time periods of less than 30 mya (Marko 2002). The same applies to other nuclear ribosomal genes (18S rRNA, 28S rRNA) used for phylogenetic analyses (Clabaut *et al.* 2005). Thus, the rapidly evolving mitochondrial COI gene is robust for the interpretation of evolutionary relationships within genera.

Among mollusks, the percentage of divergence of COI gene across the Panamic Isthmus has been studied in gastropods sister species (Hellberg 1998, Collin 2003, Latiolais *et al.* 2006, Miura *et al.* 2010), also among bivalves (Marko 2002, Lee & Foighil 2005). However, COI has not been used yet to specifically compare sister species in chitons.

According to Mayr (1969), sister species are pairs or groups of closely related species that are reproductively isolated, but are morphologically identical or nearly identical. Prior to biochemical comparisons, the criterion for proposing that a pair of chiton species were likely sister species was the demonstration of strong morphological similarity (Ferreira 1978, Bullock 1985, Lyons 1998). The putative sister species of chitons, *Stenoplax purpurascens* (Adams, 1845) (Caribbean) and *S. limaciformis* (Sowerby, 1832) (Pacific Ocean), are so similar in general form, size, shape and proportions of the valves, tegmentum sculpture and colors, shape and size of girdle scales that until recently they were classified as the same species (Ferreira 1978, Kaas & Van Belle 1980) (Fig. 1). Bullock (1985) documented subtle differences in the sculpture of the valves, the morphology of the radula, and in esthetes pore densities, which justified treating *S. purpurascens* as distinct from the older species named, *S. limaciformis*.

In a taxonomic revision of the polyplacophora of the Caribbean region, Kaas (1972) recognized that *Acanthochitona hemphilli* and *A. rhodea* as similar species in size (4-5 cm), coloration, 18 tufts with around 50 large spicules in the girdle and not rounded tegmental pustules. *Acanthochitona hemphilli* (Pilsbry, 1893) with a North Caribbean distribution and *A. rhodea* (Pilsbry, 1893) in the South (Kaas 1972). Both are so similar that Watters (1981) declared *A. rhodea* to the synonymy of *A. hemphilli*. Ferreira (1985) proposed *A. hemphilli* as a synonym of *A. rhodea* (invert the synonymy). Both researchers did not recognize morphological differences that would justify their classification as different species. *A. rhodea* was also present in the eastern tropical Pacific (Smith, 1961, Keen 1971). Ferreira (1985) considered the species complex as one, with distribution from Florida to Brazil in the Western Atlantic, and from Mexico to Peru in the Pacific. Lyons (1988) using scanning electron microscope (SEM) analysis of the valves and girdle elements, sort out their consistent morphological differences of the two Caribbean species and describe a third species: *A. ferreirai* (Lyons, 1988) as the Pacific member of the complex (Fig. 2). Lyons (1988) expressed the hypothesis of greater closeness between the *A. rhodea* and *A. ferreirai* pair.

The objective of this study was to use partial COI sequences of two pairs of chitons sister species to determine how many base substitutions have accumulated after the Panamic Isthmus separated them. With the rate of divergence of these two pairs, this gene

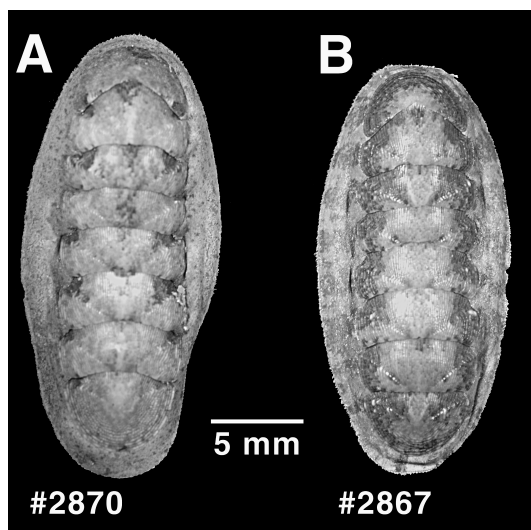


Figure 1. Dorsal view. A) *Stenoplax limaciformis* (Sowerby, 1832), CBUPRH #2870; B) *Stenoplax purpurascens* (CB Adams, 1845), CBUPRH# 2867 / Vista dorsal. A) *Stenoplax limaciformis* (Sowerby, 1832), CBUPRH #2870; B) *Stenoplax purpurascens* (CB Adams, 1845), CBUPRH# 2867

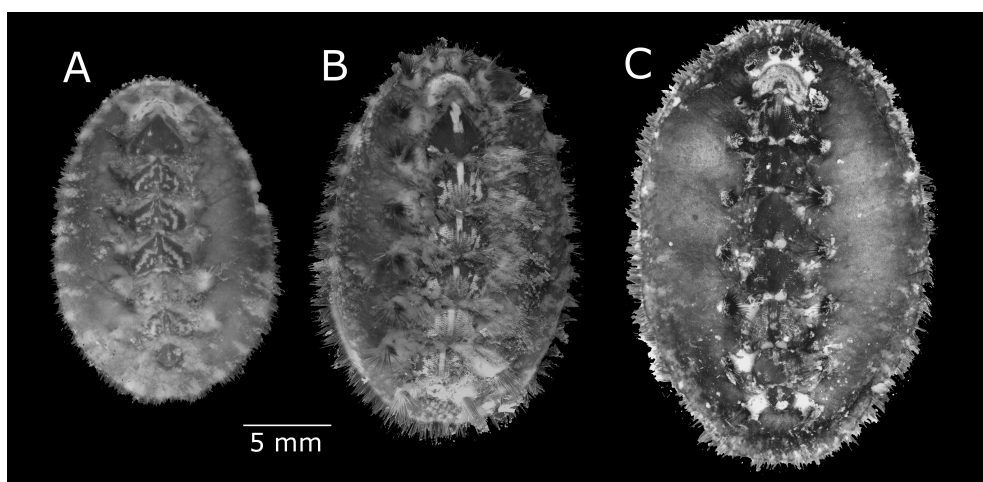


Figure 2. Dorsal view. A) *Acanthochitona ferreirai* (Lyons, 1988), CBUPRH# 3148; B) *Acanthochitona rhodea* (Pilsbry, 1893), CBUPRH# 2694; C) *Acanthochitona hemphilli* (Pilsbry, 1893), CBUPRH# 2500 / Vista dorsal. A) *Acanthochitona ferreirai* (Lyons, 1988), CBUPRH# 3148; B) *Acanthochitona rhodea* (Pilsbry, 1893), CBUPRH# 2694; C) *Acanthochitona hemphilli* (Pilsbry, 1893), CBUPRH# 2500



can be calibrated as a molecular clock. Knowledge of this divergence ratio will allow us to estimate divergence times for other chitons species.

## MATERIALS AND METHODS

### SPECIMEN'S COLLECTION

The specimens used to compare the sister species were collected in Costa Rica. Specimens of the Pacific coast were obtained in Samará Bay (9°52'29"N, 85°31'57.24"W), Guanacaste, in April 4, 2009. The specimens from the Caribbean coast were collected in Punta Puerto Vargas (9°44'40"N, 82°48'32"W), Cahuita National Park, Limón,

in April 9, 2009. The description of the collection stations and other associated species appear in Jörger *et al.* (2008), for the Pacific Coast, and in García-Ríos & Alvarez-Ruiz (2011), for the Caribbean coast. To evaluate the intraspecific variability of the gene, we included samples obtained in Puerto Rico and Colombia. Other species of the 2 compared genera collected from Bonaire, Puerto Rico and Florida were also included. Sequences from 3 Pacific species of the genus *Stenoplax*, deposited in the GenBank by other authors, were used to corroborate the close genetic affinities between putative sister species (Table 1).

**Table 1.** List of specimens and GenBank accession numbers / Lista de ejemplares y el número de acceso al GenBank

Species	Locality	Access N°	CBUPRH N°
<i>S. purpurascens</i>	Costa Rica - Caribbean, 9°44'40"N; 82°48'32"W	KC669552	2683
<i>S. purpurascens</i>	Costa Rica - Caribbean, 9°44'40"N; 82°48'32"W	KC669553	2806
<i>S. purpurascens</i>	Costa Rica - Caribbean, 9°44'40"N; 82°48'32"W	KC669554	2851
<i>S. purpurascens</i>	Puerto Rico - Caribbean, 17°56'29"N; 67°5'10"W	KC669555	2712
<i>S. purpurascens</i>	Puerto Rico - Caribbean, 17°56'23"N; 67°4'6"W	KC669556	2715
<i>S. purpurascens</i>	Puerto Rico - Caribbean, 17°56'23"N; 67°4'6"W	KC669557	2802
<i>S. purpurascens</i>	Colombia - Caribbean, 11°19'41"N; 74°4'40"W	KC669558	2867
<i>S. floridana</i>	Florida - Caribbean, 24°42'14"N; 81°9'22"W	KC669562	2804
<i>S. floridana</i>	Florida - Caribbean, 24°42'14"N; 81°9'22"W	KC669563	2852
<i>S. limaciformis</i>	Costa Rica - Pacific, 9°52'29"N; 85°31'57.24"W	KC669559	2682
<i>S. limaciformis</i>	Costa Rica - Pacific, 9°52'29"N; 85°31'57.24"W	KC669560	2871
<i>S. limaciformis</i>	Costa Rica - Pacific, 9°52'29"N; 85°31'57.24"W	KC669561	2872
<i>S. alata</i> *	Indonesia	AY377711.1	n/a
<i>S. mariposa</i> *	Eastern Pacific, 31.3°-24.15°N	EF201016.1	n/a
<i>S. conspicua</i> *	Eastern Pacific, 33.75°-29°N	EF200986.1	n/a
<i>A. rhodea</i>	Costa Rica - Caribbean, 9°44'40"N; 82°48'32"W	KC669564	2748
<i>A. hemphilli</i>	Puerto Rico - Caribbean, 17°56'37"N; 67°3'24"W	KF184977	3078
<i>A. hemphilli</i>	Puerto Rico - Caribbean, 17°56'37"N; 67°3'24"W	KF184978	3079
<i>A. hemphilli</i>	Puerto Rico - Caribbean, 17°56'23"N; 67°4'6"W	KC669566	2713
<i>A. hemphilli</i>	Bonaire-Caribbean, 12°14'27"N; 68°24'44"W	KC669567	2547
<i>A. ferreirai</i>	Costa Rica -Pacific, 9°52'29"N; 85°31'57.24"W	KC669568	2811
<i>A. ferreirai</i>	Costa Rica -Pacific, 9°52'29"N; 85°31'57.24"W	KC669569	2854
<i>A. ferreirai</i>	Costa Rica -Pacific, 9°52'29"N; 85°31'57.24"W	KC669570	2855
<i>A. balesae</i>	Colombia - Caribbean, 11°12'25"N; 74°13'49"W	KF184979	2816
<i>A. lineata</i>	Puerto Rico - Caribbean, 17°56'22"N; 67°5'13"W	KF184980	2731
<i>A. lineata</i>	Puerto Rico - Caribbean, 17°57'27"N; 67°2'32"W	KF184981	2845
<i>A. pygmaea</i>	Puerto Rico - Caribbean, 17°57'16"N; 67°3'5"W	KF184982	2810
<i>C. lata</i>	Puerto Rico - Caribbean, 17°57'27"N; 67°2'32"W	KF184983	2823
<i>C. floridanus</i>	Puerto Rico - Caribbean, 17°57'27"N; 67°2'32"W	KF184984	2809
<i>C. tuberculatus</i>	Barbados - Pokiok Rd., 32°18'26.16"N; 64°43'55.67"W	KJ909661	3068

\*COI sequences and collection data obtained from the GenBank

For the evaluation of the genetic similarity of the acanthochitonids we included other 6 species of the family: *Acanthochitona balesae* Abbot, 1954; *A. hemphilli* (Pilsbry, 1893), *A. lineata* Lyons, 1988; *A. pygmaea* (Reeve, 1847); *Choneplax lata* (Guilding, 1829) and *Cryptoconchus floridanus* (Dall, 1889). The specimens collected for this study were relaxed with ice, tied flat on a slide and preserved by total immersion in 95% ethanol. Species were identified according to Bullock (1985) and Lyons (1988). The specimens for each DNA sequence were deposited in the Colección Biológica de la Universidad de Puerto Rico en Humacao (CBUPRH). The collection numbers, location and the access number of to the sequence in GenBank are shown in Table 1. More collection data is available in the GenBank specimen information field.

#### MOLECULAR DATA COLLECTION

DNA extraction was performed using the QIAGEN DNeasy Blood & Tissue Kit. From each specimen about 10 to 15 mg of foot muscles were extracted. The DNA extracted was diluted in sterile water (200  $\mu$ l) and kept at 4°C. An average of 700 ng  $\mu$ l<sup>-1</sup> of DNA was obtained from each specimen.

The polymerase chain reaction (PCR) was performed using the extracted DNA and the COI universal primers: LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCOI 2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer *et al.* 1994). PCR reactions (25  $\mu$ l) included 0.5  $\mu$ l of the template DNA, 0.5  $\mu$ l of each primer (5 pmol  $\mu$ l<sup>-1</sup>), 2  $\mu$ l of dNTP blend (2.5 mM each), 5  $\mu$ l PCR buffer (5X) (Promega), 2.5  $\mu$ l of 2.5 mM MgCl<sub>2</sub> (Promega), and 0.25  $\mu$ l of Taq DNA polymerase (5U  $\mu$ l<sup>-1</sup>) (Promega). The PCR reactions were carried out using an Applied Biosystems thermal cycler model 2720. Thermal cycling conditions involved an initial denaturation of 5 min at 95°C, followed by 35 cycles including denaturation at 95°C for 30 s, annealing ranging from 45 to 50°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 1 min.

The PCR products were purified by precipitation with polyethylene glycol (PEG) precipitation. DNA sequencing was performed with a multicapillary sequencer (16) ABI PRISM 3130XL (Applied Biosystems) at the Sequencing and Genotyping Facility (SGF) of the University of Puerto Rico. The sequencing cycling conditions were: 96°C for 5 min, followed by 45 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, and indefinite hold at 4°C.

#### EDITING AND ANALYSIS OF THE NUCLEOTIDE SEQUENCES

Consensus sequences were determined by aligning at least two sequences from opposite directions, using the program BioEdit 7.0.9.0 (Hall 1999). All the substitutions were corroborated using the chromatograms. The resulting sequences were aligned using ClustalW 1.4 (Thompson *et al.* 1994), included in the program BioEdit. Alignments were corrected manually and ambiguously aligned regions were not used in the analysis. Sequence comparisons between pairs were performed using the program MEGA4 (Tamura *et al.* 2007). For the determination of distance between species, the percent of difference was determined using all the available bases in equal length sequences. This was the basis for subsequent phylogenetic analyzes.

DNA saturation analysis was determined graphically by plotting the transition rate / transversion rate ratio as a function of percentage divergence (Roe & Sperling 2007). Phylogenetic analyzes and the resulting trees were prepared using the Neighbor-Joining method (Saitou & Nei 1987) with bootstrap replications (1000 replicates) (Felsenstein 1985), as implemented in MEGA4.

#### EDITING AND ANALYSIS OF THE AMINO ACID SEQUENCES

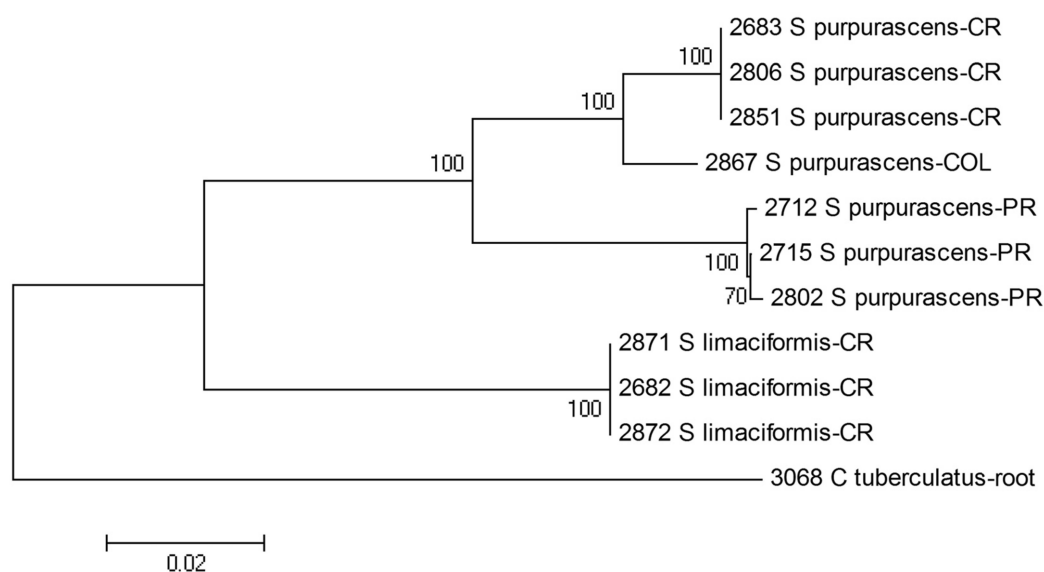
The program MEGA4 was used to determine the amino acid sequences resulting from the translation of the gene fragments, using the invertebrate mitochondrial genetic code.

#### RESULTS

The sequences of the COI gene among the sister species *Stenoplax limaciformis* and *S. purpurascens* showed 102 variable positions in the pairwise array of 642 aligned sites (about 16%). The total number of variable positions in the pairwise array of 642 aligned sites was 102 (about 16%). The comparison includes 3 specimens of *S. purpurascens* collected from three separated localities within the Caribbean: Costa Rica, Colombia and Puerto Rico. The minimum differences between the specimens of Puerto Rico and the ones from Colombia and Costa Rica are 6.2 and 6.7%, respectively. Among the specimens from Colombia and Costa Rica there is a divergence of 2.2%. The closest genetic similarity of *S. limaciformis* was with the specimens of *S. purpurascens* from Costa Rica, 11.4% divergence (0.013 SE) (Table 2, Fig. 3).

**Table 2. *Stenoplax* sister species nucleotide percent distances. Below the diagonal: percent distances calculated comparing 642 bp (initial position in the 1516 bp). Above the diagonal: transition/transversion ratio, nt= no transversion, PR= Puerto Rico, COL= Colombia, CR= Costa Rica / Porcentaje de diferencia en nucleótidos entre especies hermanas del género *Stenoplax*. Bajo la diagonal: porcentaje de diferencias en nucleótidos comparando 642 bp (iniciando en la base 1516). Sobre la diagonal: relación de transición/transversión, nt= ninguna transversión, PR= Puerto Rico, COL= Colombia, CR= Costa Rica**

	1	2	3	4	5	6	7	8	9	10
[ 1 ] #2712 <i>S. purpurascens</i> -PR		nt	nt	19.0	21.0	21.0	21.0	9.0	9.0	9.0
[ 2 ] #2715 <i>S. purpurascens</i> -PR	0.2%		nt	19.5	20.5	20.5	20.5	8.9	8.9	8.9
[ 3 ] #2802 <i>S. purpurascens</i> -PR	0.3%	0.2%		20.0	21.0	21.0	21.0	9.0	9.0	9.0
[ 4 ] #2867 <i>S. purpurascens</i> -COL	6.2%	6.4%	6.5%		nt	nt	nt	6.7	6.7	6.7
[ 5 ] #2683 <i>S. purpurascens</i> -CR	6.9%	6.7%	6.9%	2.2%		nt	nt	6.3	6.3	6.3
[ 6 ] #2806 <i>S. purpurascens</i> -CR	6.9%	6.7%	6.9%	2.2%	0.0%		nt	6.3	6.3	6.3
[ 7 ] #2851 <i>S. purpurascens</i> -CR	6.9%	6.7%	6.9%	2.2%	0.0%	0.0%		6.3	6.3	6.3
[ 8 ] #2682 <i>S. limaciformis</i> -CR	12.5%	12.3%	12.5%	12.0%	11.4%	11.4%	11.4%		nt	nt
[ 9 ] #2871 <i>S. limaciformis</i> -CR	12.5%	12.3%	12.5%	12.0%	11.4%	11.4%	11.4%	0.0%		nt
[ 10 ] #2872 <i>S. limaciformis</i> -CR	12.5%	12.3%	12.5%	12.0%	11.4%	11.4%	11.4%	0.0%	0.0%	



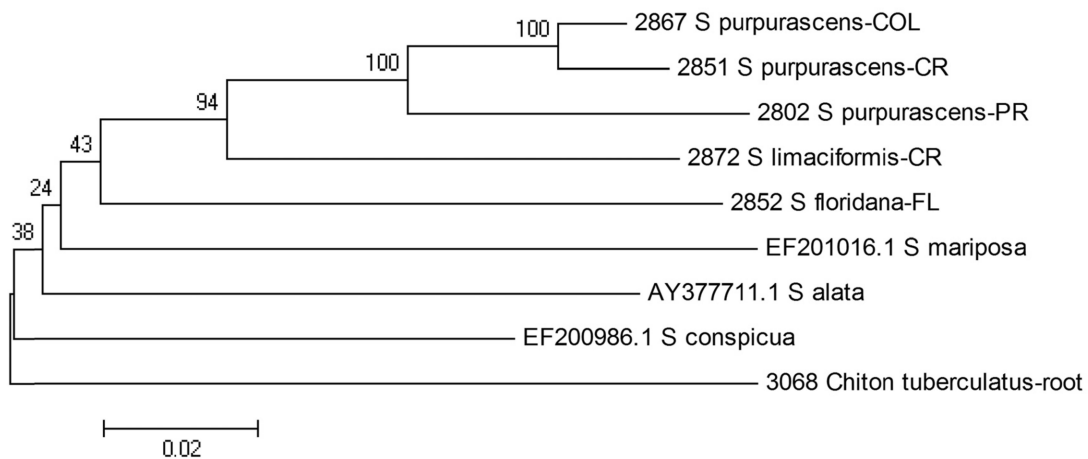
**Figure 3. Phylogenetic tree for populations of *S. purpurascens* from the Caribbean coast of Costa Rica (CR), Colombia (COL), and Puerto Rico (PR), and *S. limaciformis* from the Pacific Coast of Costa Rica; based on 642 bp of the COI gene (initial position in the 1516 bp), using nucleotide percent distance. The evolutionary relationship was inferred using the Neighbor-Joining method. Values at nodes represent the percentage of 1000 bootstrap replicates. The scale bar represent the number of substitutions per nucleotide position. Tree is rooted by *Chiton tuberculatus*. CR= Costa Rica, COL= Colombia, PR= Puerto Rico / Árbol filogenético de las poblaciones de *S. purpurascens* de la costa caribeña de Costa Rica (CR), Colombia (COL), Puerto Rico (PR) y *S. limaciformis* de la costa del Océano Pacífico de Costa Rica; basado en el porcentaje de distancia de 642 bp del gen mitocondrial COI (iniciando en la base 1516). La relación evolutiva se infiere usando el método vecino más cercano. El porcentaje de réplicas de las asociaciones entre taxones, a partir de un análisis de bootstrap de 1000, aparece cercano a las ramas. *Chiton tuberculatus* se utiliza como raíz. CR= Costa Rica, COL= Colombia, PR= Puerto Rico**

The comparison of 6 species of the genus *Stenoplax*, 4 from the Pacific and 2 from the Caribbean, showed 162 variable positions in a 515 bp matrix (31.5%). The lowest values of divergence between different species (11%) correspond to *S. purpurascens* from Costa Rica and *S. limaciformis* (Table 3). On average, the base thymine is

more abundant, with 36.7%, followed by 26.8% adenine, guanine with 20.3%, and cytosine with 16.2%. A phylogenetic tree was inferred from the data on Table 3 (Fig. 4).

**Table 3. *Stenoplax* spp. nucleotide percent distances. Below the diagonal: nucleotide percent distances calculated comparing 515 bp (initial position in the 1574 bp). Above the diagonal: transition/transversion ratio, nt= no transversion, PR= Puerto Rico, COL= Colombia, CR= Costa Rica, FL= Florida / Porcentaje de diferencias en nucleótidos entre especies del género *Stenoplax*. Bajo la diagonal: porcentaje de diferencias en nucleótidos comparando 515 bp (iniciando en la base 1574). Sobre la diagonal: relación de transición/transversión, nt= ninguna transversión, PR= Puerto Rico, COL= Colombia, CR= Costa Rica, FL= Florida**

	1	2	3	4	5	6	7	8
[1] #2802 <i>S. purpurascens</i> -PR		37	39	7.1	1.8	1.3	1.3	1.3
[2] #2867 <i>S. purpurascens</i> -COL	7%		nt	5.7	1.6	1.1	1	0.9
[3] #2851 <i>S. purpurascens</i> -CR	8%	2%		5.4	1.7	1.3	1.1	1
[4] #2872 <i>S. limaciformis</i> -CR	13%	12%	11%		1.6	1.2	1	1.1
[5] #2852 <i>S. floridana</i> -FL	16%	16%	16%	15%		1.1	1	1.4
[6] #AY377711.1 <i>S. alata</i>	15%	14%	15%	16%	17%		0.7	1.2
[7] #EF201016.1 <i>S. mariposa</i>	18%	16%	16%	18%	17%	17%		1
[8] #EF200986.1 <i>S. conspicua</i>	18%	15%	16%	16%	16%	15%	16%	



**Figure 4. Phylogenetic tree of Pacific and Caribbean *Stenoplax* spp. based on 515 bp of the COI gene (initial position in the 1574 bp), using nucleotide percent distance. The evolutionary relationship was inferred using the Neighbor-Joining method. Values at nodes represent the percentage of 1000 bootstrap replicates. The scale bar represent the number of substitutions per nucleotide position. Tree is rooted by *Chiton tuberculatus*. COL= Colombia, CR= Costa Rica, PR= Puerto Rico, FL= Florida / Árbol filogenético de poblaciones de *Stenoplax* spp. del Pacífico y Caribe, basado en el porcentaje de distancia de 515 bp del gen mitocondrial COI (iniciando en la base 1574). La relación evolutiva se infiere usando el método vecino más cercano. El porcentaje de réplicas de las asociaciones entre taxones, a partir de un análisis de bootstrap de 1000, aparece cercano a las ramas. *Chiton tuberculatus* se utiliza como raíz. COL= Colombia, CR= Costa Rica, PR= Puerto Rico, FL= Florida**

The graph of percent genetic distance against the ratio of transitions: transversions, made with COI sequences of all *Stenoplax* specimens considered here suggests poor saturation before reaching 14% of divergence (Fig. 5).

An analysis of the amino acid sequence encoded by the compared gene fragments revealed that from a total of 171 amino acids, there were five variations among the 6 species compared. No differences were found in the pair *S. purpurascens* and *S. limaciformis* (all substitutions are silent). On the other hand, *S. floridana* differs by one

amino acid (position 158 of our sequence) of the previous mentioned pair. The substitution of isoleucine (position 158) to valine in *S. floridana* (northern Caribbean species) was shared by *S. alata* (Indonesia) and *S. mariposa* (Eastern Pacific). *Stenoplax conspicua* differed from the pair of putative sister species in one amino acid (position 134). The amino acid at that position, phenylalanine, was shared by the other two species of the Pacific, but not with *S. limaciformis* (Table 4).

**Table 4. Comparison of a partial sequence of the amino acids of the COI in some species of the *Stenoplax* genus / Comparación de una secuencia parcial de los aminoácidos del COI en algunas especies del género *Stenoplax***

#2802_S_purpurascens-PR	LIRAEELGQPG	ALLGDDQLYN	VIVTAHAFVM	IFFLVMPMMI	GGFGNWLVP
#2867_S_purpurascens-COL	.....	.....	.....	.....	.....
#2851_S_purpurascens-CR	.....	.....	.....	.....	.....
#2872_S_limaciformis-CR	.....	.....	.....	.....	.....
#2852_S_floridana-FL	.....	.....	.....	.....	.....
#AY377711.1_S_alata	.....	.....	.....	.....	.....
#EF201016.1_S_mariposa	.....	.....	.....	.....	.....
#EF200986.1_S_conspicua	.....	.....	.....	.....	.....
#2802_S_purpurascens-PR	MLGAPDMAFP	RLNNSFWLL	PPALCLLLGS	AAVESGVGTG	WTVYPPLASN
#2867_S_purpurascens-COL	.....	.....	.....	.....	.....
#2851_S_purpurascens-CR	.....	.....	.....	.....	.....
#2872_S_limaciformis-CR	.....	.....	.....	.....	.....
#2852_S_floridana-FL	.....	.....	.....	.....	.....
#AY377711.1_S_alata	.....	.....	.....	.....	.....
#EF201016.1_S_mariposa	.....	.....	.....	.....	.....
#EF200986.1_S_conspicua	.....	.....	.....	.....	.....
#2802_S_purpurascens-PR	IAHAGGSVDL	AIFSLHLAGV	SSILGAVNFI	TTVLNMRWKG	MQMERLPLFV
#2867_S_purpurascens-COL	.....	.....	.....	.....	.....
#2851_S_purpurascens-CR	.....	.....	.....	.....	.....
#2872_S_limaciformis-CR	.....	.....	.....	.....	.....
#2852_S_floridana-FL	.....	.....	.....	.....	.....
#AY377711.1_S_alata	.....	.....	.....	F.....	.....
#EF201016.1_S_mariposa	.....SA.....	.....	.....	F.....	.....L.....
#EF200986.1_S_conspicua	.....	.....	.....	F.....	.....
#2802_S_purpurascens-PR	WSVKITAILL	LLSLPVLGG	I		
#2867_S_purpurascens-COL	.....	.....	.		
#2851_S_purpurascens-CR	.....	.....	.		
#2872_S_limaciformis-CR	.....	.....	.		
#2852_S_floridana-FL	.....V..	.....	.		
#AY377711.1_S_alata	.....V..	.....	.		
#EF201016.1_S_mariposa	.....V..	.....	.		
#EF200986.1_S_conspicua	.....	.....	.		

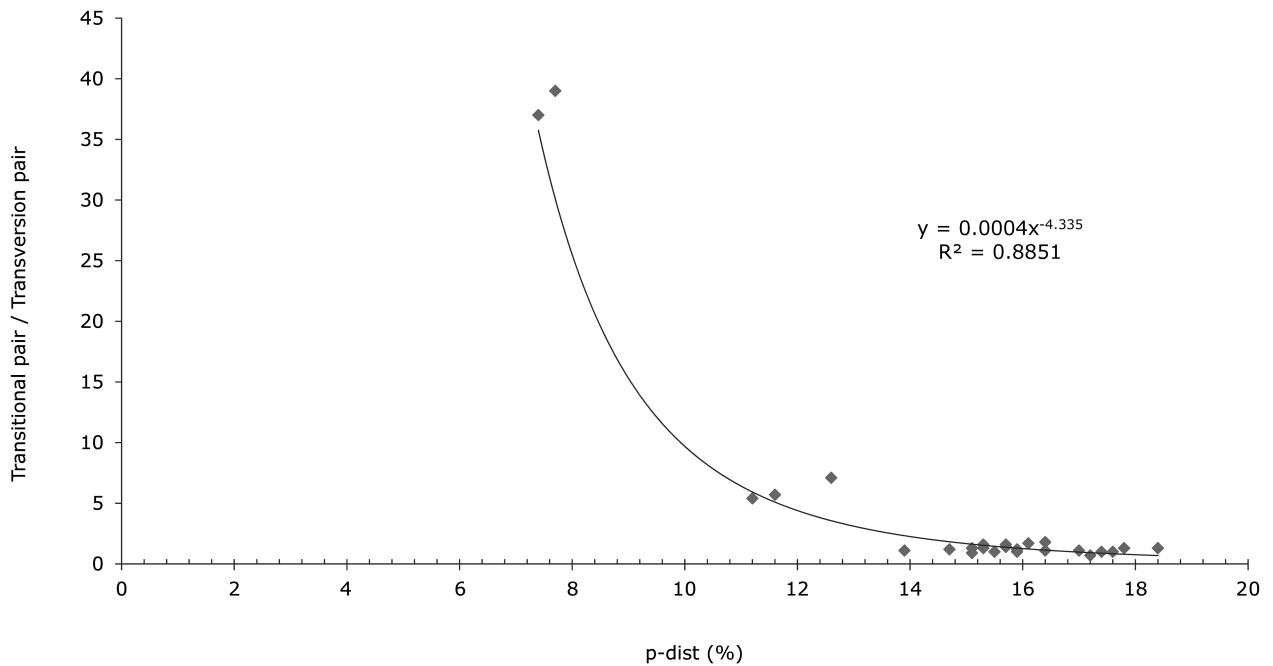


Figure 5. COI gene saturation analysis using percent distance versus transition/transversion ratio of *Stenoplax* spp. / Análisis de saturación del gen COI, utilizando el porcentaje de distancia versus la razón de transición/transversión de *Stenoplax* spp.

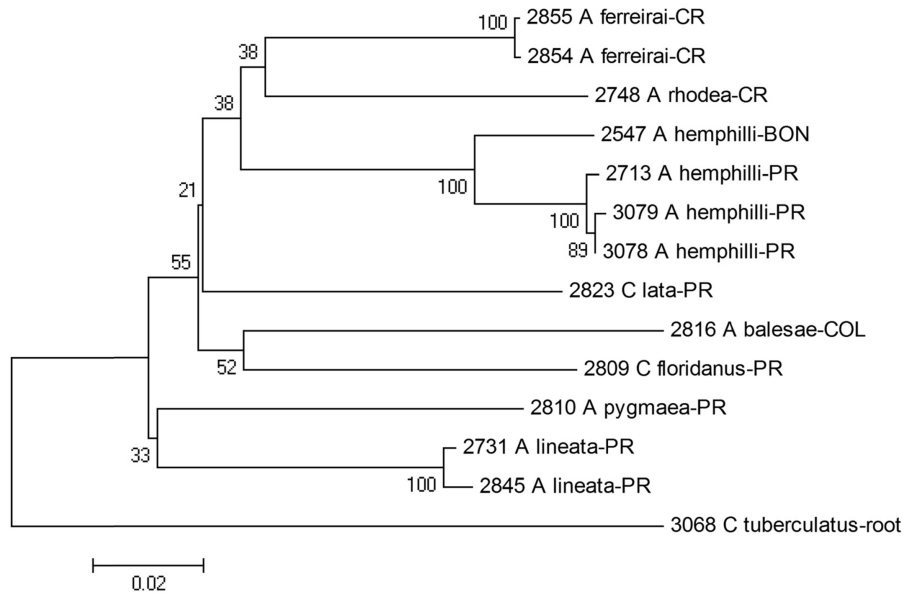


Figure 6. Phylogenetic tree for selected Acanthochitonidae species from the Caribbean and *A. ferreirai* from the Pacific coast of Costa Rica; based on 551 bp of the COI gene (initial position in the 1562 bp), using nucleotide percent distance. Values at nodes represent the percentage of 1000 bootstrap replicates. The scale bar represent the number of substitutions per nucleotide position. Tree is rooted by *Chiton tuberculatus*. CR= Costa Rica, BON= Bonaire, PR= Puerto Rico / Árbol filogenético de especies caribeñas de la familia Acanthochitonidae y *A. ferreirai* de la costa del Pacífico de Costa Rica, basado en el porcentaje de distancia de 551 bp del gen mitocondrial COI (iniciando en la base 1574). La relación evolutiva se infiere usando el método vecino más cercano. El porcentaje de réplicas de las asociaciones entre taxones, a partir de un análisis de bootstrap de 1000, aparece cercano a las ramas. *Chiton tuberculatus* se utiliza como raíz. CR= Costa Rica, BON= Bonaire, PR= Puerto Rico

The comparison of the sequence divergence of 551 base pairs of COI in 8 species of 3 genus of the family Acanthochitonidae, shown in Fig. 6. The total number of variable positions in the comparison matrix was also large, 162 (29.9%). On average, the base thymine is more abundant, with 42.3%, followed by guanine (23.0%), adenine (20.5%), and cytosine with 14.2%.

The acanthochitonids COI sequences compared encoded a total of 183 amino acids. There were only 2 variations in the amino acid sequence among the 9 species compared. *Crytoconchus floridanus* differs by one amino acid (position 162 of our sequence), *Acanthochitona pygmaea* and *A. balesae* differs in the 143 amino acid. The other 6 species showed identical amino acid sequence fragments (silent nucleotide substitutions) (Table 5).

**Table 5. Comparison of a partial sequence of the amino acids of the COI in some species in the family Acanthochitonidae / Comparación de una secuencia parcial de los aminoácidos del COI en algunas especies de la familia Acanthochitonidae**

#2816_A_balesae	ALSLLRIRAEI	GQPGALLGDD	QLYNVIVTAH	AFVMIFFLVM	PMMIGGFGNW
#2854_A_ferreirai	.....	.....	.....	.....	.....
#2855_A_ferreirai	.....	.....	.....	.....	.....
#2713_A_hemphilli	.....	.....	.....	.....	.....
#3078_A_hemphilli	.....	.....	.....	.....	.....
#3079_A_hemphilli	.....	.....	.....	.....	.....
#2731_A_lineata	.....	.....	.....	.....	.....
#2845_A_lineata	.....	.....	.....	.....	.....
#2810_A_pygmaea	.....	.....	.....	.....	.....
#2547_A_hemphilli-BON	.....	.....	.....	.....	.....
#2748_A_rhodea	.....	.....	.....	.....	.....
#2823_C_lata	.....	.....	.....	.....	.....
#2809_C_floridanus	.....	.....	.....	.....	.....
#2816_A_balesae	LVPLMLGAPD	MAFPRLNNMS	FWLLPPSLCL	LLASGAVESG	VGTGWTVYPP
#2854_A_ferreirai	.....	.....	.....	.....	.....
#2855_A_ferreirai	.....	.....	.....	.....	.....
#2713_A_hemphilli	.....	.....	.....	.....	.....
#3078_A_hemphilli	.....	.....	.....	.....	.....
#3079_A_hemphilli	.....	.....	.....	.....	.....
#2731_A_lineata	.....	.....	.....	.....	.....
#2845_A_lineata	.....	.....	.....	.....	.....
#2810_A_pygmaea	.....	.....	.....	.....	.....
#2547_A_hemphilli-BON	.....	.....	.....	.....	.....
#2748_A_rhodea	.....	.....	.....	.....	.....
#2823_C_lata	.....	.....	.....	.....	.....
#2809_C_floridanus	.....	.....	.....	.....	.....
#2816_A_balesae	LAGNVAHAGG	SVDLAIFSLH	LAGVSSILGA	VNFITTVLNM	RWVGMQLERM
#2854_A_ferreirai	.....	.....	.....	.....	.S.....
#2855_A_ferreirai	.....	.....	.....	.....	.S.....
#2713_A_hemphilli	.....	.....	.....	.....	.S.....
#3078_A_hemphilli	.....	.....	.....	.....	.S.....
#3079_A_hemphilli	.....	.....	.....	.....	.S.....
#2731_A_lineata	.....	.....	.....	.....	.S.....
#2845_A_lineata	.....	.....	.....	.....	.S.....
#2810_A_pygmaea	.....	.....	.....	.....	.G.....
#2547_A_hemphilli-BON	.....	.....	.....	.....	.S.....
#2748_A_rhodea	.....	.....	.....	.....	.S.....
#2823_C_lata	.....	.....	.....	.....	.S.....
#2809_C_floridanus	.....	.....	.....	.....	.S.....
#2816_A_balesae	PLFVWSVKIT	AVLLLLSLPV	LAGGITMLLT	DRN	
#2854_A_ferreirai	.....	.....	.....	.....	.....
#2855_A_ferreirai	.....	.....	.....	.....	.....
#2713_A_hemphilli	.....	.....	.....	.....	.....
#3078_A_hemphilli	.....	.....	.....	.....	.....
#3079_A_hemphilli	.....	.....	.....	.....	.....
#2731_A_lineata	.....	.....	.....	.....	.....
#2845_A_lineata	.....	.....	.....	.....	.....
#2810_A_pygmaea	.....	.....	.....	.....	.....
#2547_A_hemphilli-BON	.....	.....	.....	.....	.....
#2748_A_rhodea	.....	.....	.....	.....	.....
#2823_C_lata	.....	.....	.....	.....	.....
#2809_C_floridanus	.....	.I.....	.....	.....	.....

A comparison of divergence of the 590 bp COI sequence in 8 specimens of the 3 morphologically and genetically similar *Acanthochitona* species was done. Three of the specimens compared belong to the species *A. ferreirai* (Pacific coast of Costa Rica), 4 specimens of *A. hemphilli* (Pilsbry 1893), 3 from Puerto Rico and one of Bonaire, and a specimen of *A. rhodea* from the Caribbean coast of Costa Rica. Sequence comparisons between the 3 species of *Acanthochitona*, revealed that the smallest distance found between *A. rhodea* (Caribbean Costa Rica) was with the specimens of *A. ferreirai* of the Costa Rica Pacific coast (10.5 to 10.4%, 0.002 SE). While the distances to specimens of *A. hemphilli* of Bonaire and Puerto Rico were 12.4 and 13.2-13.6%, respectively. Among the specimens of *A. hemphilli* of Bonaire and Puerto Rico we found a distance of 4.8-5.1% (Table 6 and Fig. 6).

## DISCUSSION

The phylogenetic comparison between species of the genus *Stenoplax* (Fig. 4) and the comparison of the 171 amino acids sequence (Table 4) is consistent with the hypothesis that *S. purpurascens* and *S. limaciformis* are sister's species. Equally, the comparison of the sister species *Acanthochitona hemphilli*, *A. rhodea* and *A. ferreirai* (Fig. 6) identifies the species *A. rhodea* (Southern Caribbean) and *A. ferreirai* (Tropical Eastern Pacific) to be genetically closer (Table 6). This supports the hypothesis expressed by Lyons (1988: 86) of greater closeness between this pair.

The graph of genetic percent distance against the transition/transversion ratio, shows a saturation of about

14% of divergence (Fig. 5). This is similar to the 11% reported for red algae (Sherwood *et al.* 2010) and much less than 20% (K2p distance) for species of shrimps of the genus *Macrobrachium* (Zhang *et al.* 2009). This limits the use of the COI gene as a molecular clock in chitons to divergences of less than 14%, margin that allows the calibration of the sister species compared in this study.

Using as separation time of the populations 3.1 mya, based on the minimum estimated time that the Caribbean and Pacific faunas are thought to have been isolated by the Panamic Isthmus, then this assumed vicariance date would lead to a calculated divergence ratio of 3.7% / mya in *Stenoplax* (Table 2) and 3.4% / mya in *Acanthochitona* (Table 6). These values correspond to 4.1 K2p in *Stenoplax* and 3.6 K2p in *Acanthochitona* (Kimura 1980 method, as implemented by MEGA4).

Divergence values of 3.4 to 3.7% bp / mya calculated for these chiton species are of similar magnitude to those reported for shrimps of the genus *Penaeus* (3%, Baldwin *et al.* 1998), *Diadema* echinoderms (2.9 to 4.2%, Lessios *et al.* 2001) *Tripneustes*, *Diadema* and *Meoma* (3.5%, Lessios *et al.* 2003) and higher than the 2.6% calculated for the gastropod *Echinolittorina* (Williams & Reid 2004) and 1.8 to 2.2% in *Eucidaris* and *Echinometra* urchins (Birmingham & Lessios 1993). The heterogeneous molecular evolutionary rates among taxa are well documented (*e.g.*, Kumar 2005, Ward 2009, Morrison *et al.* 2004). The differences in generation time of each species could explain some of those differences. However, some of these divergence rates have been qualified as overestimates (Marko 2002), because not all marine

**Table 6. *Acanthochitona* sister species nucleotide percent distances. Below the diagonal: nucleotide percent distances calculated comparing 590 bp (initial position in the 1550 bp). Above the diagonal: transition/transversion ratio, nt= no transversion, CR= Costa Rica, PR= Puerto Rico, BON= Bonaire / Porcentaje de diferencias en nucleótidos entre especies hermanas del género *Acanthochitona*. Bajo la diagonal: porcentaje de diferencias en nucleótidos comparando 590 bp (iniciando en la base 1550). Sobre la diagonal: relación de transición/transversión, nt= ninguna transversión, CR= Costa Rica, PR= Puerto Rico, BON= Bonaire**

	1	2	3	4	5	6	7	8
[1] #2855 <i>A. ferreirai</i> -CR		nt	nt	3.1	1.5	1.6	1.6	1.7
[2] #2811 <i>A. ferreirai</i> -CR	0.0%		nt	3.1	1.5	1.6	1.6	1.7
[3] #2854 <i>A. ferreirai</i> -CR	0.2%	0.2%		3.1	1.6	1.6	1.7	1.8
[4] #2748 <i>A. rhodea</i> -CR	10.5%	10.5%	10.4%		1.7	1.6	1.6	1.6
[5] #2713 <i>A. hemphilli</i> -PR	10.7%	10.7%	10.9%	13.6%		nt	nt	14.0
[6] #3078 <i>A. hemphilli</i> -PR	11.0%	11.0%	11.2%	13.2%	0.3%		nt	13.0
[7] #3079 <i>A. hemphilli</i> -PR	11.2%	11.2%	11.4%	13.4%	0.5%	0.2%		13.5
[8] #2547 <i>A. hemphilli</i> -BON	10.7%	10.7%	10.9%	12.4%	5.1%	4.8%	4.9%	



organisms might be able to keep genetic connectivity through a shallower seaway, where conditions of high sedimentation characteristic of mangroves existed prior to the end of the closing (Knowlton & Weigt 1998).

The use of fossil record data for the calibration of molecular clocks in transisthmian sister species, has shown that times of separation for the mollusks associated with reefs and rocky coasts took place between 5.1-10.1 mya (Marko 2002). If we assume that the chitons populations were also isolated with the interruption of environments with hard-bottom and low sedimentation rates, the adjusted calibration for *Stenoplax* will be 2.2 to 1.1% / mya and 2.0 to 1.0% for *Acanthochitona*. These values of divergence ratio are similar to the 1.2% calculated for clams in the family Arcidae (Marko 2002) and 2.4% for gastropods of the genus *Tegula* (Hellberg & Vacquier 1999). Both estimates belong to mollusks that inhabit rocky bottoms, as the chitons. For multiple pairs of shrimps in the genus *Alpheus* a rate of 1.4% / mya was inferred by Knowlton & Weigt (1998).

The genetic distances between individuals of the same species in different localities of the Caribbean showed values up to 8.0% in *S. purpurascens* and 4.9% in *A. hemphilli*. The specimens compared are not the most distant species within the genus; therefore higher intraspecific differences can be expected (Tables 2 and 4). These divergence values exceed the distance used to define some species (Avice 2000). Distances greater than those are found among many chitons species, according to Kelly & Eernisse (2008). These divergence values suggest a reduced gene flow between the populations compared here, and support the early separation hypothesis.

Although rafting on floating objects as adults is possible, it is more likely that most of the dispersion depends on distances traveled during the pelagic larval stages, for at least free spawning species. Because chiton larvae are lecithotrophic (*i.e.*, non-feeding), even those from free spawned gametes probably have a relatively short pelagic stage. Overall, the pelagic phase of the larval stage can last from hours to more than a week (Strathmann & Eernisse 1987). In culture, *Mopalia* spp. larvae were observed to settle in 5 to 10 days (Leise 1984, Eernisse 1988). It is assumed that the development of larvae in tropical waters is faster, which should further reduce the population connectivity (Kelly & Eernisse 2007). Reported larval settling time for *Chiton tuberculatus* is 3 days, and 2 days for *Acanthopleura granulata* (Lewis 1960). Bullock

(1985) postulated that the pattern of distribution of chiton species in the Caribbean suggests that larvae must only remain in the plankton for a short time. Heath (1899 in Bullock 1985) reported that larvae of *Stenoplax heathiana* complete their free-swimming stage within 15 min to 3 h after hatching, but this species does not free spawn gametes, as most common chitons do. Instead, the female lays sticky benthic egg masses, and larvae develop within these, only emerging at a late larval stage already capable of remaining benthic without any pelagic stage. A short pelagic stage could be consistent with the high values of divergence between populations that we found, but reproduction has not been studied in any of these species. In order to calibrate molecular clocks with sister species, specimens should be collected on the coasts of the Isthmus, thus to avoid the error introduced by the variability of the gene within the geographic distribution of the species.

Using the phylogenetic trees for *Stenoplax* species (Fig. 3) and Acanthochitonidae family species (Fig. 6) we can establish that the distances between sister's species are very small. The morphological similarity between these species is not the product of convergence evolution of distantly related species. However, the low values of the bootstraps test for higher clades do not allow inferences on the evolutionary relationship among all the species represented. Such analysis would require the inclusion of other taxonomic gene and a more complete representation of these genera.

The rise of the Isthmus of Panama altered the chemical and physical conditions of the oceans that were separated (Glynn 1972). This event detoured the North Atlantic Equatorial Current to the north, intensifying the flow of currents in the Caribbean and the Gulf of Mexico (Berggren & Hollister 1974, Lessios 2008), creating warmer and saltier conditions throughout the Caribbean and western Atlantic (Jackson *et al.* 1993). The change created a favorable scenario for the northward movement of tropical marine species. For species with limited dispersal and movement, such as chitons, the closure of the isthmus should have promoted colonization and speciation processes. As this is a recent geologic event, we expect that these speciation processes are not always reflected in distinctive morphological adaptations. The use of the COI gene can aid to identify possible cryptic species, and our calibration can estimate divergence times for these new species.

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