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PHYLOGENETIC RELATIONSHIPS AMONG SPECIES OF *PROTOTHACA* FROM PANAMA BASED ON CYTOCHROME C OXIDASE I (COI) SEQUENCES.

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ABSTRACT: The genus *Protothaca* (Bivalvia: Veneridae) represents a commercially important group of clams with a wide distribution in the Pacific coast of America and Asia. According to The Integrated Taxonomic Information System (ITIS), there are nine accepted species (http://www.itis.gov/); nevertheless the number of recognized species varies based on the sources revised. In this study, a molecular approach was used to phylogenetically analyze five species belonging to the genus *Protothaca*. A fragment of 550 bp from the mitochondrial cytochrome oxidase I gene was analyzed from *P. asperrima*, *P. columbiensis*, *P. grata*, *P. mcgyntyi* and *P. jedoensis*. The COI sequences revealed seven haplotypes for *P. asperrima*, four for *P. columbiensis* and two for *P. grata*. Bayesian analysis showed two major well supported clusters consisting of (1) genus-*Protothaca* clade and (2) a clade with rest of the members of Veneridae. All the species of *Protothaca* clustered together with the exception of *P. jedoensis*. Intraspecific genetic differences in *P. asperrima* were very high at 17-18 %; *P. columbiensis* and *P. grata* specimens had differences in the range of 0,18-0.36 % and 0,92 % respectively. Interestingly, *Protothaca asperrima* haplotypes A and H showed the highest level of intraspecific genetic distances. Considering the traditional controversies in classification, the level of COI divergence in *P. asperrima* haplotypes and the differences observed in other genera within the family Veneridae, we propose a revision of the species including elevating the rank of the two distinct clusters of *P. asperrima* to closely related species status.

Key words: Genetic difference, COI, mtDNA, *P. asperrima*, Veneridae.

RESUMEN: El género *Protothaca* (Bivalvia:Veneridae) representa un grupo de almejas comercialmente importantes con una amplia distribución en las costas del Pacífico de América y Asia. De acuerdo con el Sistema Integrado de Información Taxonomica (ITIS), hay nueve especies aceptadas; sin embargo el número de especies reconocidas varía en función de las fuentes revisadas. La taxonomía actual del género se basa en la morfología de la concha y la clasificación sigue siendo controvertida. En este estudio, se utilizó un enfoque molecular para analizar filogenéticamente cinco especies pertenecientes al género *Protothaca*. Se analizó un fragmento de 550 pb del gen mitocondrial de citocromo oxidasa I de *P. asperrima*, *P. columbiensis*, *P. grata*, *P. mcgyntyi* y *P. jedoensis*. Las secuencias de COI revelaron siete haplotipos de *P. asperrima*, cuatro para *P. columbiensis* y dos para *P. grata*. El análisis bayesiano mostró dos grupos principales, bien soportados consistentes en (1) clado del género-*Protothaca* y (2) un clado con el resto de los miembros de Veneridae. Todas las especies de *Protothaca* agruparon juntas con la excepción de *P. jedoensis*. La diferencia genética intraespecífica en P. asperrima fué bastante alta entre 17-18%; Las diferencias entre especímenes de *P. columbiensis* y *P. grata* estuvieron en el rango de 0.18-0.36% y 0.92% respectivamente. Curiosamente, los haplotipos A y H de *Protothaca* asperrima mostraron el nivel más alto de distancias genéticas intraespecífica. Teniendo en cuenta las controversias tradicionales en la clasificación, el nivel de divergencia COI en los haplotipos de *P. asperrima* y las diferencias observadas en otros géneros dentro de la familia Veneridae, proponemos una revisión de las especies, incluyendo la elevación del rango de los dos grupos diferentes de P. asperrima al estatus de especies estrechamente relacionadas.

Palabras clave: Diferencia genética, COI, mtDNA. *P. aspérrima*, Veneridae

INTRODUCTION

The Veneridae, commonly known as "venus clams", is the most diverse and recent bivalve family, with approximately 170 genera and over 800 extant, presumably valid, species. The most widely used classification of the family Veneridae was proposed by KEEN (1969). The 12 subfamilies recognized by Keen do not reflect genetic relationships and this classification is considered inadequate (CANAPA *et al*. 2003). The family was recently restructured based on morphological and molecular data into 14 nominal subfamilies by MIKKELSEN *et al*. (2006); however the authors underscored the need for revision at lower taxonomic levels. The family is one of the least understood and comprises of poorly defined bivalvian taxa, despite including species of major economic importance in commercial, recreational, and native fisheries (SARTORI *et al*. 2008).

Veneridae classification has been historically controversial in terms of taxon placement because of undue emphasis on morphological characters. Modern phylogenetic studies on other bivalve groups have shown that morphological traits frequently do not support currently accepted classifications (GRAF 2000; LYDEARD *et al*. 2000). The presence of the anterior lateral tooth in the left valve of *Ameghionomya antigua* (venerid), for example, has been used as a criterion to place it in the Venerinae genus *Venus* (FISCHER-PIETTE & VUKADINOVICH 1977) whereas specimens with the missing tooth have been classified as species in the Chioninae genus *Protothaca* (AGUIRRE & FARINATI 2000) or as a subgenus of *Chione* (FRIZZELL 1936). Molecular data based on nuclear and mitochondrial DNA sequences placed the disputed morphotypes into separate clades: Venerinae *s. novo* and Chioninae *s.s.* (KAPPNER & BIELER 2006).

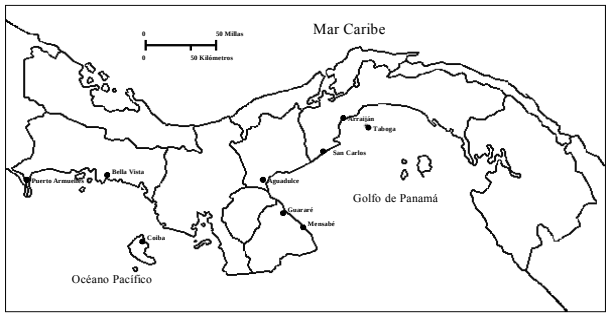

Fig. 1. Map of the Republic of Panama. The collection localities are represented by black dots. The scale map are shown in miles and kilometers.

The genus *Protothaca* (DALL 1902) is included within the subfamily Chioninae, family Veneridae. The genus comprises of validated and invalidated species or species with interim taxonomical status. Although, an increasing number of taxonomic studies on bivalves have been reported based on molecular data, only a few species of the genus *Protothaca* including *P. staminea* and *P. mcgyntyi* (KAPPNER & BIELER 2006) were considered before this study. The genus *Protothaca* occurs mainly in the Pacific and most of the species live along the west coast of America from Baja California to Peru (PALACIOS *et al*. 1986), in Japan and in New Zealand (OLSSON 1961). The genus includes species of economic importance such as *P. staminea* and *P. asperrima*. The latter is harvested by local fishermen for their own consumption or sale at local markets in Costa Rica and Panama (MOFFAT & NICHOL ENGINEERS 2005).

Traditionally different species names and taxonomic status have been assigned to *P. asperrima* sensu SOWERBY (1835). These names include: *Venus asperrima*, *Venus histrionica*, *Venus intersecta* and *Tapes tumida*, (used by SOWERBY 1835, 1853). OLSSON (1961) classified *Nioche* (Nioche) *asperrima asperrima* and *Nioche* (Nioche) *asperrima histrionica* as two subspecies of *P. asperrima*; this situation was changed by KEEN (1971) who classified the subspecies recognized by OLSSON (1961) as a single species. The taxonomic status of *P. asperrima* is similar to many other bivalves and mollusks, separated by some authors and unified by others as a consequence of using only morphological criteria that are not always adequate to resolve lower taxonomical categories (PEEK *et al*. 1997; TAYLOR & GLOVER 2006).

The present study utilized the nucleotide sequence variation of cytochrome oxidase I gene (COI) to analyze the genetic differences in some species of the genus *Protothaca* and to determine the phylogenetic relationships among the genus and among other members of the Veneridae family. COI fragments were sequenced for *P. asperrima*, *P. columbiensis*, *P. grata* and have been deposited in Genbank.

MATERIALS AND METHODS

Samples

The specimens of *Protothaca columbiensis* SOWERBY (1835) *Protothaca grata* SAY (1830) and *Protothaca asperrima* SOWERBY (1835) used in this study were

collected in the Pacific coast of Panamá (Figure 1). The collected specimens were identified using characters described by KEEN (1971), VAUGHT and CUNNINGHAN (1989) and HUBER (2010). The specimens were deposited in the Museum of Malacology of University of Panama (MMUP). The voucher numbers and the collection locations are summarized in Table 1.

DNA Extraction

Total genomic DNA was extracted from adductor muscle according to MEDINA & WALSH (2000). Briefly, approximately 20 mg of adductor muscle tissue was gently homogenized in 700 µL of NET buffer (NaCl 150 mM, EDTA 10 mM, Tris-HCl 10 mM, pH 8.0), containing 1% SDS and 100 µg/mL of proteinase K and incubated at 65°C in a water bath for 2 h. The samples were subjected to two phenol:chloroform (3:1) and one chloroform:isoamyl alcohol (24:1) extractions followed by precipitation with 300 mM sodium acetate and 2.5-3 volumes of absolute ethanol. Genomic DNA was resuspended in TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) and treated with RNase A (50 µg/µL) at 37°C for 1h followed by a chloroform:isoamyl alcohol (24:1) extraction and absolute ethanol precipitation. The precipitated DNA pellet was resuspended in TE buffer and DNA quality was evaluated by 1% agarose electrophoresis using K562 DNA marker as standard (Promega).

TABLE 1. Taxa studied with the corresponding accession number. The location and voucher number are shown. The species which were sequenced in this study are in bold.

Species	Location	Voucher	GenBank accession
1. Clausinella punctifera	Cape Verde	FMNH 310503	DQ458477
2. Venus verrucosa	France: Paris Fish Market	FMNH 310506	DQ458499
3. Tawera spissa	New Zealand	FMNH 310514	DQ458493
4. Chione subimbricata	Panama: Pacific	FMNH 310508	DQ458475
5. Lirophora mariae	Panama: Pacific	FMNH 310509	DQ458483
6. Callista chione	Senegal	FMNH 306218	DQ458473
7. Macrocallista squalida	Panama: Pacific	FMNH 310517	DQ458485
8. Dosinia victoriae	Australia	FMNH 306202	DQ458479
9. Katelysia sp.1	Australia: Fish Market	AMNH 311619	DQ184824
10. Ruditapes decussatus	Portugal: Fish Market	FMNH 306189	DQ184830
11. Protothaca mcgyntyi	Panama: Pacific	FMNH 310511	DQ458490
12. Protothaca jedoensis	China	H.L.Cheng 200505011	DQ399397
13. Protothaca jedoensis	China	B.P.Pan,S.P.Zhang Ga1-24	EU118008
14. Protothaca asperrima (A1)	Arraiján, Panama	MMUP 3504	EU165041
15. Protothaca asperrima (A2)	Mensabé, Los Santos, Panama	MMUP18516	EU165042
16. Protothaca asperrima (A3)	Aguadulce, Coclé, Panama	MMUP2101	EU165043
17. Protothaca asperrima (A4)	Coiba, Veraguas, Panama	MMUP 7306	EU165044
18. Protothaca asperrima (H1)	Arraiján, Panama	MMUP18355	EU165045
19. Protothaca asperrima (H2)	Aguadulce, Coclé, Panama	MMUP18047	EU165046
20. Protothaca asperrima (H4)	Taboga, Panamá, Panama	MMUP18385	EU165047
21. Protothaca columbiensis (C1)	San Carlos, Panamá	MMUP25439	EU165048
22. Protothaca columbiensis (C2)	Guararé, Los Santos, Panama	MMUP24123	EU165049
23. Protothaca columbiensis (C3)	Bella Vista, Chiriquí, Panama	MMUP24300	EU165050
24. Protothaca columbiensis (C5)	Arraiján, Panama	MMUP21873	EU165051
25. Protothaca grata (G2)	Aguadulce, Coclé, Panama	MMUP18165	EU165052
26. Protothaca grata (G4)	Puerto Armuelles, Chiriquí, Panama	MMUP18002	EU165053

MMUP: Museum of Malacology, University of Panama.

PCR Amplification, Molecular Cloning and Sequencing

PCR amplifications were performed according to the procedure described by BALDWIN *et al*. (1996). The universal COI primers (FOLMER *et al*. 1994) were used to amplify the COI fragment: LCO 1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO 2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). PCR was performed in a total reaction volume of 25µL that consisted of 2.5µL of 10x PCR buffer (Promega), 3.0 µL of $MgCl_2$ (25 mM), 0.25 µL of dNTPs (25mM), 0.6 µL of each primer (45 µM), 0.6 µL of Taq DNA polymerase (5 u/µL), 15.45 µL of nuclease free sterile water and 2.0 µL (~50 ng) of DNA. The PCR conditions consisted of denaturation for 1 minute at 95ºC followed by 35 cycles of 15 s at 94ºC, 1 minute at 54ºC, and 1 minute at 72ºC, with a final extension step of 7 minutes at 72ºC. PCR products of approximately 658 bp were visualized by agarose electrophoresis and purified using the Wizard® PCR Preps DNA Purification System (Promega). Due to difficulties obtaining sequences from direct PCR products, we decided to clone COI fragments into pGEM®-T Easy Vector System kit (Promega). Chemically competent *E. coli* DH5á cells were prepared and transformed cells were plated onto LB agar containing X-gal and IPTG (Sambrook & Russell, 2001). Plasmid DNA prepared from clones were verified using either PCR or digestion with *Eco*RI. Recombinant plasmids were purified using the Wizard® Plus Miniprep DNA Purification System (Promega) and sequenced using M13 primers and BigDye® terminator cycle sequencing ready reaction mix (Applied Biosystems) and run on ABI PRISM® 310 sequencer.

Sequence Alignment and Phylogenetic Analyses

Electropherograms with nucleotide composition for the sequences generated in this study were examined and multiple DNA sequences were assembled into a format useable for phylogenetic analysis using Sequencher 4.7 (Genecodes Corp., Ann Arbor, MI). Sequences with a Phred value of 25 or higher were used for analysis and both strands were sequenced for confirmation. Because the sequences used in the phylogenetic analyses have different lengths, we selected a 550 bp common region of the gene. COI sequences obtained from Genbank for comparisons with the *Protothaca* specimens used in this study are listed in Table 1. The sequences aligned using ClustalW (Thompson *et al*. 1994) were used to construct phylogenetic trees using three different methods. Maximum parsimony and Bayesian analyses were done using PAUP beta 4.0 program (SWOFFORD 2002) and MrBayes program, respectively (RONQUIST & HUELSENBECK 2003). We also used neighbor-joining (NJ) with the Kimura 2-parameter model of substitution (REF) to create an additional tree and calculated the genetic distances comparable with the DNA barcode identification systems (RATNASINGHAM & HEBERT 2007). For maximum parsimony analysis, searches were done using the heuristic search option. Starting trees were obtained via stepwise addition and branch swapping was implemented using the tree-bisection-reconnection (TBR) option. Bayesian Metropolis coupled Markov chain Monte Carlo (MCMCMC) estimation of phylogeny was done using GTR + Ã (nst=6, rates=gamma). Two simultaneous independent runs were performed for a total of 5,000,000 generations to ensure that the MCMCMC had converged on stable log likelihood. Out of 1000 trees generated, 250 were excluded as "burn in". Posterior probabilities equal to and above 95% were considered as significant.

RESULTS

A total of 26 COI sequences from the family Veneridae were analyzed of which 13 were generated in this study. The taxa included members of the subfamilies Venerinae, Chioninae, Pitarinae, Dosiniinae and Tapetinae. With the exception of Dosiniinae at least two species from each subfamily were considered for a total of 15 species corresponding to eleven genera. Each genus was represented by at least one species. Since we are interested in determining intrageneric genetic divergences in *Protothaca*, five species of the genus were included in the study.

Sequence alignment and analysis

Sequences obtained in this study have been deposited in Gen Bank (Accession numbers EU165041-EU165053). The COI alignment for 26 representative sequences contained 550 bp with 317 variable sites, of which 264 were parsimony informative. When multiple individuals produced identical sequences, only one sequence was included in the analysis. An indel of three nucleotides was observed in all *Protothaca* sequences with the exception of *P. jedoensis* when compared with the COI sequences from the others Veneridae members used in this analysis.

COI divergences

KIMURA (1980) two-parameter and p-distances were

calculate from 550 bp COI sequences. A clear stepwise increase was found in intraspecific and interspecific distances. No overlap was found between pairwise intra- and interspecific distances. Distances between some genera overlapped with some interspecific pairwise comparisons. The *P. asperrima* A1 lineage showed a distance of 28% when compared with two different genera *Chione subimbricata* and *Tawera spissa* and 29% with *P. columbiensis*.

Pairwise intraspecific genetic distances in *P. asperrima* were 0 -18 %; 0 % for *P. columbiensis* and 0-1% for *P. grata*. *Protothaca* interspecific genetic distances range from 23-43 %, more than one order of magnitude higher than intraspecific distances.

The COI sequences revealed seven haplotypes for *P. asperrima*, four for *P. columbiensis* and two for *P. grata*. The haplotypes of *P. asperrima* differ by single substitutions in no more than three points. All except one of the substitutions are transitions. The haplotype A3 is characterized by an A-T transversion, which was the only difference that produced an amino acid change (Threonine-Serine). The *P. asperrima* haplotypes were separated into two groups designated by H (for *histrionica*) and A (for *asperrima*). Four single transitions haplotypes were observed in *P. columbiensis* without any change in amino acid sequences. The two haplotypes observed in *P. grata* were represented by three single transitions dispersed at different positions along the COI sequence. The alignment at protein level of these two haplotypes revealed a change in amino acid sequence from serine to lysine.

The genetic distances among genera of Chioninae subfamily ranged from 19-28%. However the observed distance between *P. columbiensis and Chione subimbricata,* representing genera from two different subfamilies (Chioninae and Venerinae) was 24%, exceeding the distance value observed for different genera from Chioninae subfamily.

Phylogenetic Analysis

Phylogeny trees were constructed using Parsimony, Bayesian and Neighbor-Joining approaches. *Callista chione,* a member of subfamily Pitarinae, was chosen as an outgroup based on the results of phylogenetic analyses of Veneroidea (Mikkelsen *et al*. 2006). Parsimony analysis resulted in a tree with 1185 steps and a consistency index of 0,4353 (data not shown). The three methods used generated congruent results since the tree topologies were similar. The Bayesian tree (Figure 2) shows two major clades: a *Protothaca* clade and a clade comprising other Veneridae species included in the study. All *Protothaca* species grouped together with highly supported branches with the exception of *P. jedoensis* sequence (DQ399397), which grouped with other Veneridae genera. Within the *Protothaca* clade, there is a subclade consisting of *P. mcgyntyi* and *P. asperrima* and another clade including specimens of *P. columbiensis, P. jedoensis* (EU118008) and *P. grata*. Specimens of *Protothaca asperrima* Sowerby (1835) formed two subgroups within this subclade.

The second major clade consisted of the species *Clausinella punctigera*, *Venus verrucosa*, *Chione subimbrincata*, *Lirophora mariae*, *Tawera spissa*, *Dosinia victoriae*, *Katelysi*a sp. and *Ruditapes decussatus*. Within this clade the species from each subfamily usually group together.

DISCUSSION

Cytochrome C Oxidase I (COI), the genetic marker proposed for DNA barcoding, has been used to determine phylogenetic relationship among bivalves because it is a fast evolving mitochondrial gene and it has been successfully used in mollusk systematics and provides resolution from family to subspecies level (Kappner & Bieler 2006; Wood *et al*. 2007; Hare & Weinberg 2005; Canapa *et al*. 2003; Matsumoto 2003). We used sequence divergence based on COI to clarify the controversial taxonomy of *P. asperrima* and to determine the phylogenetic relationships among members of this genus with other genera from the Veneridae family.

The phylogenetic analyses showed a tendency of members from the same subfamily to cluster together with some exceptions. Some taxa including *L. mariae* and *C. subimbricata* that are expected to group with the Chioninae subfamily clustered in a separated clade with Venerinae and others subfamilies. However, the clade support values for these clusters were relatively lower than others branches in both, Bayesian and Maximum Parsimony trees.

Phylogenetic analyses based on Bayesian inference showed two major clades with well-supported bootstrap

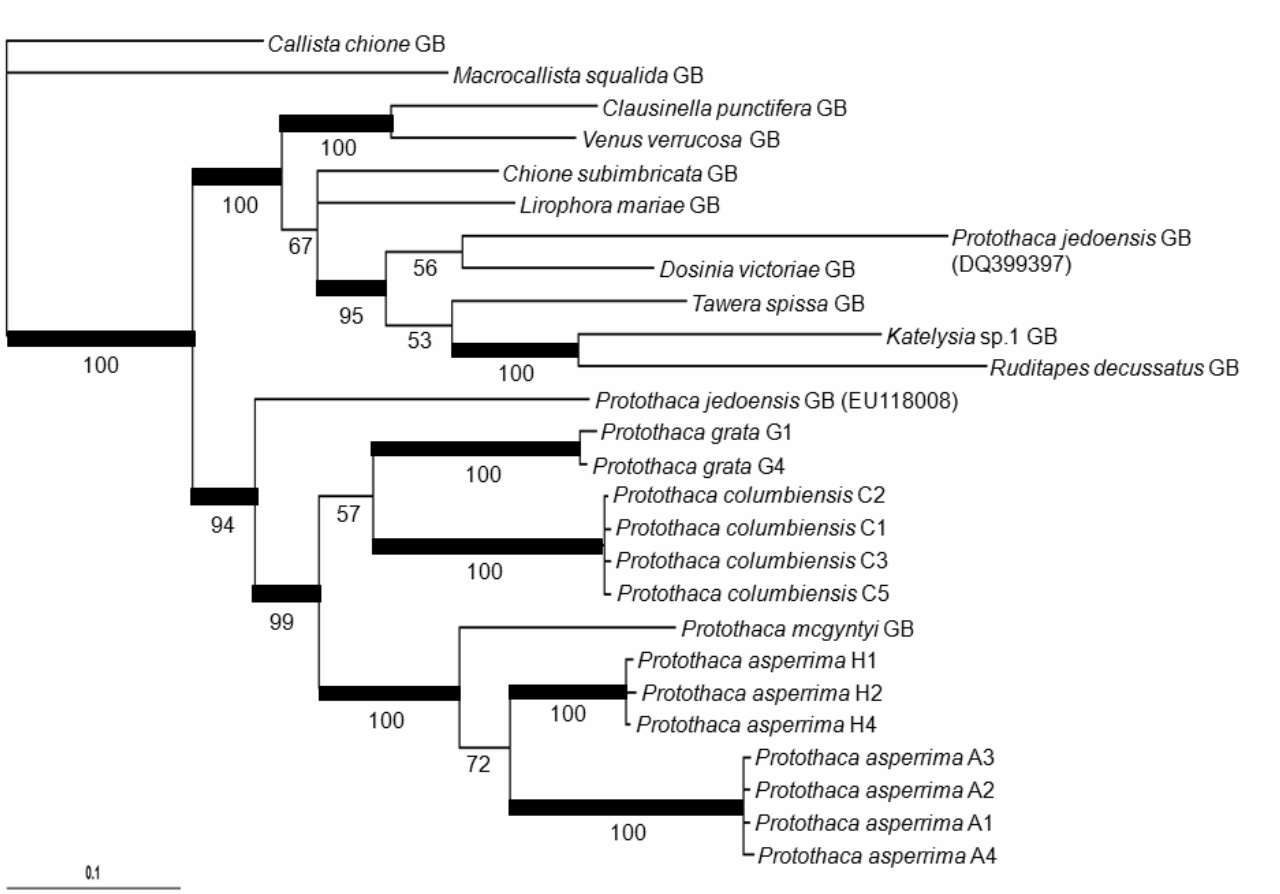

Fig. 2. Bayesian tree analyzed using 5 million generations. Clade support values of 80 and above are shown. Clades with more than 95% support values are indicated with a solid line. GB = Sequences downloaded from GenBank.

values. The first clade comprised members of subfamilies Venerinae, Tapetinae, Dosiniinae and Chioninae. The other clade includes only species belonging to the genus Protothaca, subfamily Chioninae (Figure 2).

Specimens included in the *P. asperrima* group formed two well supported clades in trees constructed using different methods clearly indicating a distinction between haplotypes A and H. In contrast, *P. columbiensis* and *P. grata* individuals did not show multiple clusters within the species. Our finding that *P. asperrima* haplotypes H clustered separately from *P. asperrima* haplotypes A is more in agreement with OLSSON (1961), who recognized two *P. asperrima* subspecies based on anatomical conchological data, than with the classification proposed by KEEN (1971). Olsson considered shell color differences and some variations in the ribs as morphological criteria for the separation (Figure 3).

Based on shell characteristic OLSON (1961) distinguishes two *P. asperrima* subspecies: *P. asperrima asperrima* and *P. asperrima histrionica*. The shell in *P. asperrima asperrima* is either chalky white or sometimes irregularly blotched with brown color, while *P. asperrima histrionica* has a ground color usually white, with three or more, discontinuous, radial rows of brown or finger-like markings. The shell in *P. asperrima asperrima* is subcircular to subovate, generally coarse, moderately convex, sculptured by rather coarse, rasplike radial costae but in *P. asperrima histrionica* the shell is usually smaller, ovate to oblong, plump and solid. The average length is seldom over 45 mm. The costal intervals in *P. asperrima*

asperrima are deep, narrow grooves. Both the costals and their intervals are crossed by strong, corded concentrics producing a pattern like that of a coarse lattice. The sculpture in *P. asperrima histrionica* is produced by small, cordlike costae crossed by evenly spaced, raised, corded concentrics producing a finely reticulated pattern.

The specimens with A haplotype exhibited the typical morphological characteristics of *P. asperrima asperrima* and the specimens with H haplotype, the morphological characters of *P. asperrima histrionica*. These results indicate a clear correspondence between morphological and genetic differences at least in *P. asperrima*.

The magnitude of divergence found between *P. asperrima* A and H haplotypes is out of the range reported for intraspecific COI divergence. The genetic distances between *P. asperrima* haplotypes A and H was considerably higher (17-18 %) than expected for marine bivalves COI haplotypes. There are no reports of this magnitude of divergence at intraspecific level for COI in bivalves. For example, sequence divergence between COI haplotypes of pearl oyster *Pinctada mazatlantica* was estimated to be 0,12-1,3 % (ARNAUD *et al*. 2000). BALDWIN *et al*. (1996) reported genetic distances of 0,65 % for COI sequences in two varieties of *Dreissena bugensis*.

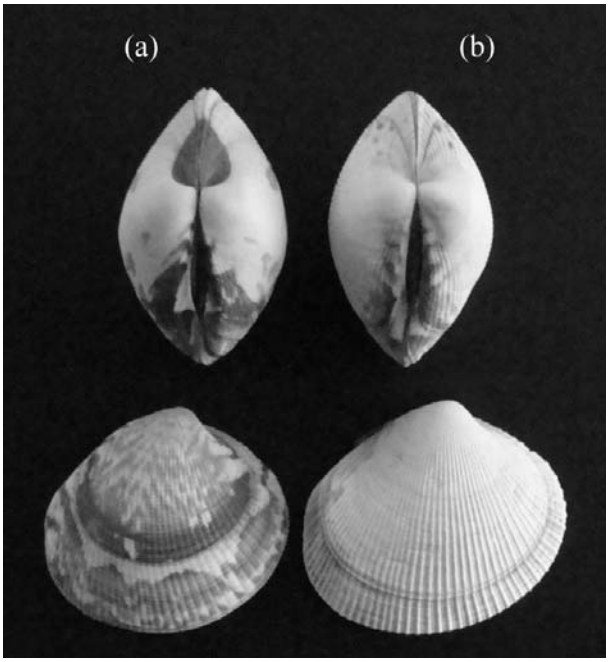

Fig. 3: (a) *Protothaca histrionica* SOWERBY, 1835; (b) *Protothaca asperrima* (SOWERBY, 1835)

The levels of intraspecific COI divergence in *P. columbiensis* and *P. grata* in this study were 0,1 and 1 %, respectively. The genetic distances between *P. asperrima* haplotypes are two orders of magnitude higher than the observed among other species of *Protothaca*. Although we have no reports of such level of COI divergence in bivalves it is important to mention that ROMERO & RAMÍREZ (2011) found 18 % of divergence for COI sequences on haplotypes of *Systrophia helicycloides*, a terrestrial gastropod.

Interestingly, the percentage of difference obtained as a complement of percentage of similarity using BOLD engine (www.barcodinglife.org) for some species from Chioninae (*Chione fasciata* vs *C. punctifera, Lirophora paphia* vs *L. mariae, Placamen berryi* vs *P. flindersi*); Dosiniinae (*Dosinia victoriae* vs *Dosinia* sp.1); Tapetinae (*Katelysi rhytiphora* vs *K. scarlina*) and Venerinae (*Venus casina* vs *V. verrucosa*) subfamilies ranged between 12-17 %, very similar to the range observed for *P. asperrima* haplotypes.

The range of genetic variation observed in *Protothaca* species was considerably high. BALDWIN *et al*. (1996) found that interspecific genetic distances between *Dreissena bugensis* and *D. polymorpha* ranged from 16-17%. Similar results were reported by KOJIMA *et al*. (1995) in three *Calyptogena* species with COI distances ranging from 10-17%.

Pairwise comparisons between *P. jedoensis* (DQ399397) with any of the *Protothaca* species included in this study range from 30 to 43%; a value that notably exceeds the differences found in other pairwise comparisons among *Protothaca* species (17-29%) and, in some cases, higher than certain intergeneric comparisons.

Surprisingly, the two *P. jedoensis* COI sequences (EU118008 and DQ399397), were separated in two different clades. *P. jedoensis* (DQ399397) clustered -in all trees- in a clade separate from the *Protothaca* clade (data not shown). *P. jedoensis* (DQ399397) grouped with a species of the subfamily Venerinae (*Tawera spissa*) in Parsimony and NJ trees and with a species of the subfamily Dosiniinae (*Dosinia victoriae*) in the Bayesian tree. When we used the identification engine from BOLD Systems and Blast search for the *P. jedoensis* COI sequence (DQ399397), the output showed 100 % of specimen similarity with *Mactra veneriformis* (Mactridae) but not with *Protothaca jedoensis* (EU118008) as we would expect.

When we performed a Blast search using EU118008 (which corresponds to another COI sequence from *P. jedoensis* in Genbank), some homology was observed with *Clausinella isabellina*, a member of the same subfamily (Chioninae) as *P. jedoensis*. The discrepancy among the only two *P. jedoensis* sequences deposited in the Genbank and the significant homology of *P. jedoensis* EU118008 sequence with members of the same subfamily could imply that the DQ399397 sequence does not correspond to an individual of this species.

There is no clear relationship in Pairwise comparisons among genetic and geographical distances in *P. asperrima* haplotypes. In some cases the larger distances corresponding to genetic distances slightly higher than the genetic distances observed in haplotypes separated by short distances such as A1-A3 and A1-A4. But in others cases such as H1-H4 and H2-H4, both have the same genetic distances in spite of which geographical distance in H1-H4 is 20 kilometers apart and H2-H4 is 120 kilometers. This inconsistency could be explained on the basis of complexity of marine environments and mobility of marine species in different developmental phases (ZHAN *et at*. 2009).

Considering the traditional classification controversies in some members of genus, the level of COI divergence in *P. asperrima* haplotypes and the data obtained from reproductive studies showing two reproductive peacks (PALACIOS *et al*. 1986; LÓPEZ *et al*. 2005), we propose a revision of the taxonomical status of species belonging to the genus *Protothaca* .

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