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MOLECULAR PHYLOGENY OF THE NERITIDAE (GASTROPODA: NERITIMORPHA) BASED ON THE MITOCHONDRIAL GENES CYTOCHROME OXIDASE I (COI) AND 16S rRNA

Filogenia molecular de la familia Neritidae (Gastropoda: Neritimorpha) con base en los genes mitocondriales citocromo oxidasa I (COI) y 16S rRNA

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ABSTRACT
The family Neritidae has representatives in tropical and subtropical regions that occur in a variety of environments, and its known fossil record dates back to the late Cretaceous. However there have been few studies of molecular phylogeny in this family. We performed a phylogenetic reconstruction of the family Neritidae using the COI (722 bp) and the 16S rRNA (559 bp) regions of the mitochondrial genome. Neighbor-joining, maximum parsimony and Bayesian inference were performed. The best phylogenetic reconstruction was obtained using the COI region, and we consider it an appropriate marker for phylogenetic studies within the group. Consensus analysis (COI +16S rRNA) generally obtained the same tree topologies and confirmed that the genus *Nerita* is monophyletic. The consensus analysis using parsimony recovered a monophyletic group consisting of the genera *Neritina*, *Septaria*, *Theodoxus*, *Puperita*, and *Clithon*, while in the Bayesian analyses *Theodoxus* is separated from the other genera. The phylogenetic status of the species from the genus *Nerita* from the Colombian Caribbean generated in this study was consistent with that reported for the genus in previous studies. In the resulting consensus tree obtained using maximum parsimony, we included information on habitat type for each species, to map the evolution by habitat. Species of the family Neritidae possibly have their origin in marine environments, which is consistent with conclusions from previous reports based on anatomical studies.

Keywords: Colombian Caribbean, mitochondrial genes, mtDNA, *Nerita*, *Neritina*, radiation.

RESUMEN
La familia Neritidae cuenta con representantes en regiones tropicales y subtropicales adaptadas a diferentes ambientes, con un registro fósil que data para finales del Cretáceo. Sin embargo no se han realizado estudios de filogenia molecular en la familia. En este estudio se realizó una reconstrucción filogenética de la familia Neritidae utilizando las regiones COI (722 pb) y 16S rRNA (559 pb) del genoma mitocondrial. Se realizaron análisis de distancias de Neighbor-Joining, Máxima Parsimonia e Inferencia Bayesiana. La mejor reconstrucción filogenética fue mediante la región COI, considerándola un marcador apropiado para realizar estudios filogenéticos dentro del grupo. El consenso de las relaciones filogenéticas (COI+16S rRNA) permitió confirmar que el género *Nerita* es monofilético. El consenso del análisis de parsimonia reveló un grupo monofilético formado por los géneros *Neritina*, *Septaria*, *Theodoxus*, *Puperita* y *Clithon*, mientras que en el análisis bayesiano *Theodoxus* se encuentra separado de los otros géneros. El resultado en las especies del género *Nerita* del Caribe colombiano fue consistente con lo reportado para el género en estudios previos. En el árbol resultante del análisis de parsimonia se sobrepuso la

información del hábitat de cada especie, para mapear la evolución por hábitat. Se obtuvo como resultado que las especies de la familia Neritidae posiblemente tengan su origen en un ambiente marino, siendo congruente con lo reportado en estudios anatómicos realizados anteriormente.

Palabras clave: Caribe colombiano, genoma mitocondrial, ADNmt, *Nerita*, *Neritina*, radiación.

INTRODUCTION

The Neritimorpha (Neritopsina) comprises more than 450 extant species, with a fossil record reported from the Middle Devonian ca 375 million years ago, but possibly as early as Ordovician (Kano *et al.*, 2002). The families Neritidae, Phenacolepadidae, Neritopsidae, Helicinidae, Ceresidae, Proserpinidae, Hydrocenidae, and Titiscaniidae are included in this group (Thompson, 1980; Ponder and Lindberg, 1997; Ponder, 1998).

Among gastropods, Neritimorpha has had one of the greatest adaptive radiation processes. The group has invaded marine, fresh water, and groundwater environments, and exhibits a great variety of forms (Kano *et al.*, 2002). Snails with spiral (several families) or conical forms (Hydrocenidae), with or without opercula, and even slugs that do not develop shells (Titiscaniidae) are included in this group. Some species can be found in terrestrial environments such as those belonging to the families Helicinidae, Ceresidae, Proserpinidae, and Hydrocenidae, whereas other species, namely those in Neritidae, can be found in freshwater and estuarine environments (Thompson, 1980; Ponder, 1998).

The Neritidae has representatives in tropical and subtropical regions, adapted to different environments, and exhibits morphological modifications in various habitats (Holthuis, 1995; Kano *et al.*, 2002). This family seems to have its origins in the sea (Kano *et al.*, 2006). About 100 species of the genus *Nerita* live on marine and intertidal rocks. Species of the genus *Smaragdia* are found in seagrass areas. However, a higher diversity of Neritidae occurs in freshwater and estuarine waters, in terms of both numbers of genera and of species. Worldwide, 200 species comprise the genera *Neritodryas*, *Clithon*, *Vittina*, *Neritina*, *Neripteron* and *Septaria* (Kano *et al.*, 2002; Kano *et al.*, 2006). Members of the family Neritidae are relatively well represented in the fossil record, dating from the end of the Cretaceous (Bandel, 2008; Frey and Vermeij, 2008).

The evolutionary relations within the family Neritidae have not been well studied, although important studies have been conducted on various genera. For the genus *Nerita*, there is a very complete analysis of the molecular phylogeny and biogeography of the group in the tropics, using the COI and 16S rRNA genes of the mitochondrial genome and the ATPS subunit of the nuclear genome (Frey and Vermeij, 2008; Frey, 2010a). Other studies reconstructed the evolutionary history of the genus *Theodoxus* and its distribution across the Tethys Sea, using COI and 16S rRNA genes (Bunje and Lindberg, 2007). Studies have also been conducted on the genus *Neritina* using the COI gene, to analyze the phylogenetic distribution of different reproductive strategies (Kano, 2009).

Using anatomical data of species in different genera within Neritopsina, Holthuis (1995), performed a phylogenetic reconstruction of the group, and proposed a phylogeny based on 57 morphological characters. Other studies have used species of the family Neritidae to resolve the phylogeny of Neritimorpha (Neritopsina) and the evolutionary history of the group using nuclear and mitochondrial markers (Kano *et al.*, 2002; Aktipis and Giribet, 2010; Castro and Colgan, 2010).

In this study we performed a phylogenetic analysis of the family Neritidae using COI and 16S rRNA regions of the mitochondrial genome, and included species from the Colombian Caribbean. Additionally we reconstructed the evolution of the family using habitat types.

MATERIALS AND METHODS

Study Area

The Colombian Caribbean is located in the northwestern corner of South America (Fig. 1), and includes a coastline of 1937 km, a land area of 7037 km^2, and territorial waters of 532162 km^2 (Posada *et al.*, 2010). A great variety of environments is represented, including estuarine, marine, and freshwater ecosystems, and the region exhibits a high diversity of organisms.

Collection and Identification of Samples and Sequences

Nine species of neritid snails were collected from different habitats of the Colombian Caribbean (Table 1) and were preserved in 96 % ethanol. Samples were identified using morphological taxonomic keys and catalogs (Russell, 1941; Díaz and Puyana, 1994; Yidi and Sarmiento, 2010).

Sequences of the COI and 16S rRNA (16S) regions of additional species of the family Neritidae were obtained from GenBank, and were stored with the program MEGA 5 (Tamura *et al.*, 2011), and used in conjunction with the species collected and analyzed from the Colombian Caribbean region. Table 2 shows accession numbers, locality, and habitat for each species downloaded from GenBank. Habitat and locality information were complemented by a literature review. The following total numbers of species by genus were obtained: *Puperita* (1), *Clithon* (3), *Nerita* (44), *Neritina* (4), *Septaria* (2) and *Theodoxus* (3).

DNA Extraction, Amplification and Sequencing

DNA was extracted from the tissue of the foot of each species, using the DNA easy tissue extraction kit (QIAGEN, Valencia, California). The cytochrome oxidase I and 16S rRNA regions of the mitochondrial genome were amplified for each species. PCRs were performed on a total volume of 25 µl. Reactions contained 2,5 µl of 10X buffer, 1 µl of $MgCl_2$ (25 mM), 0,5 µl dNTPs (1 mM), 0,5 µl of Taq polymerase (5 U/mL) (BIOLINE), 2 µl of each primer (10 mM) and 1 µl of DNA.

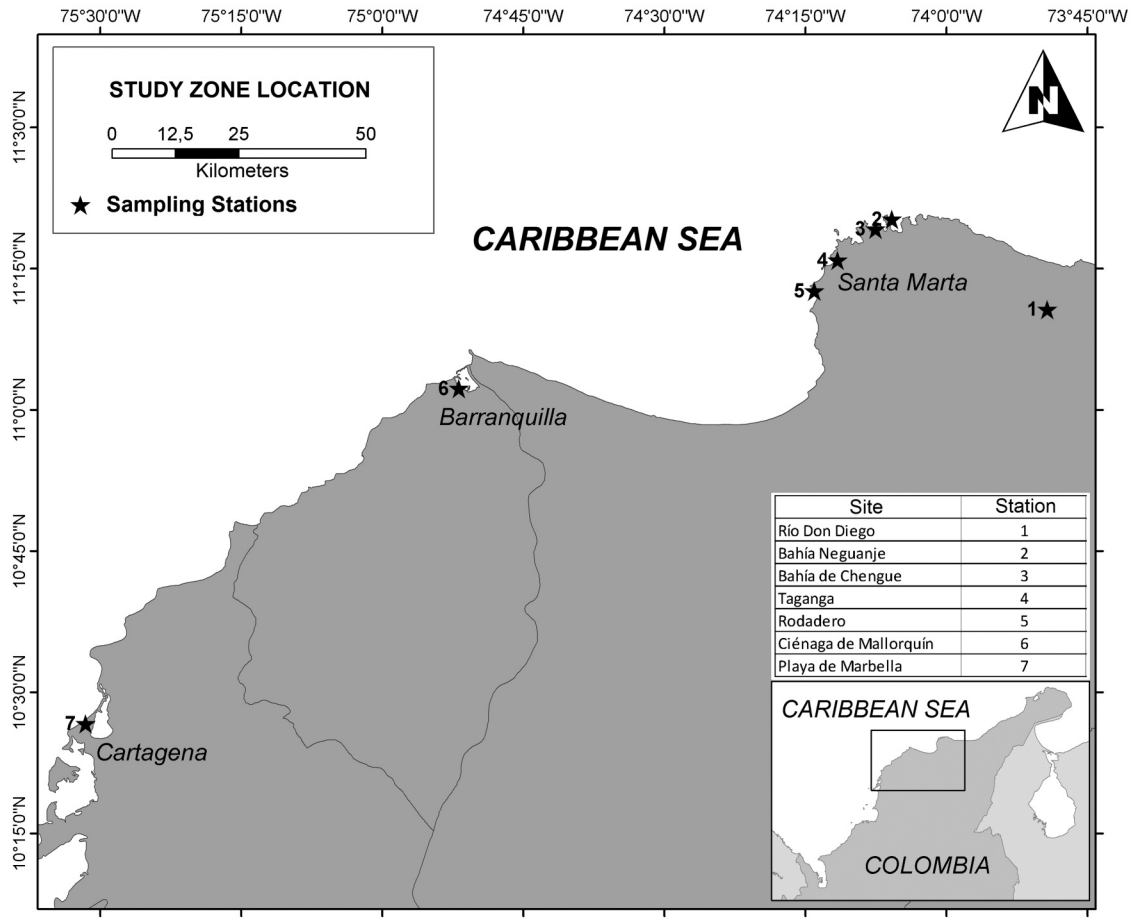

Figure 1. Study area showing the sampling sites.

Amplifications were performed in an Eppendorf gradient PCR thermocycler with the following primer combinations: [COI (HCO2198 5' - 3' TAAACTTCAGGGTGACCAAAAAATCA) and (LCO1490 5' - 3' GGTCAACAAATCATAAAGATATTGG) (Folmer *et al.*, 1994)]; [16S (16Sar 5' - 3' CGCCTGTTTATCAA AAACAT) and (16Sbr 5' - 3' CCGGTCTGAACTCAGATCACGT) (Palumbi, 1996)].

PCR conditions for each gene varied, but generally the amplification consisted of denaturation at 95 °C for 1:00 min, 35 cycles of denaturation at 95 °C 00:15 s, annealing for the COI gene was 46-51 °C, and for the 16S gen was 51-57 °C for 1:00 min, and 1:30 min extension at 72 °C, followed by 5:00 min final extension at 72 °C. PCR optimization for each template involved the variation of $MgCl_2$ concentration and annealing temperature. To remove unincorporated primers and dNTPs before sequencing, double-stranded PCR products were purified using the nucleic acids purification kit of MACHEREY-N. Both strands of the PCR product were sequenced. Primer sequences were removed from the start and the end of the obtained sequence and sequence ambiguities were resolved by comparing the electropherograms using the program BioEdit v. 7.0.5.3 (Hall, 1999). The sequences were submitted to GenBank and are available under Accession numbers JX646654 to JX646671 (Table 1).

Sequence Alignment

CLUSTAL X (Thompson *et al.*, 1997) was used to align the edited sequences and the sequences of the species of Neritidae obtained from GenBank. Representatives of six different genera out of the 16 genera reported for the family were thus included.

Alignments were performed using MEGA (v.5) (Tamura *et al.*, 2011). We used MEGA (v.5) to align the COI gene, because this approach can translate the protein-coding nucleotide sequences using the invertebrate mitochondrial genetic code, align the resulting amino acid sequences using Clustal, and then create a nucleotide sequence alignment using the amino acid alignment as a guide.

The Clustal settings for the COI gene were: pairwise alignment parameters: gap open penalty = 10, extension penalty = 0,1; multiple alignment parameters: gap open penalty = 10, extension penaly = 0,2; protein weight matrix = Gonnet 250; residue specific penalties = on; hydrophobic penalties = on; gap separation distance = 4; end gap separation = off; negative

Table 1. Locality and GenBank accession number of the sequences obtained in this study.

Species	Location	N	W	Habitat	COI	16S
Nerita fulgurans	Magdalena (Bahía Neguanje)	11°20'11"	74°5'46"	Marine	JX646664	JX646655
Nerita peloronta	Bolívar (Playa de Marbella)	10°26'37"	75°31'30"	Marine	JX646665	JX646656
Nerita tessellata	Magdalena (Taganga)	11°15'52"	74°11'33"	Marine	JX646663	JX646654
Nerita versicolor	Magdalena (El Rodadero)	11°12'36"	74°14'02"	Marine	JX646666	JX646658
Neritina meleagris	Atlántico (Ciénaga de Mallorqui)	11°2'14"	74°51'49"	Estuarine	JX646671	JX646662
Neritina piratica	Atlántico (Ciénaga de Mallorqui)	11°2'14"	74°51'49"	Estuarine	JX646669	JX646660
Neritina punctulata	Magdalena (Río Don Diego)	11°10'38"	73°49'14"	Freshwater	JX646667	JX646657
Neritina usnea	Atlántico (Ciénaga de Mallorqui)	11°2'14"	74°51'49"	Estuarine	JX646670	JX646661
Neritina virginea	Atlántico (Ciénaga de Mallorqui)	11°2'14"	74°51'49"	Estuarine	JX646668	JX646659

Table 2. Species of the family Neritidae from which COI and 16SrRNA sequences were obtained from GenBank, including NCBI accession number. The habitat and locality were taken from the publication of the Author. A-S (Author Sequences) 1. Aktipis and Giribet,2010, 2. Frey and Vermeij, 2008, 3. Bunje and Lindberg, 2007.

Species	# Acc. GenBank CO1	# Acc. GenBank 16S	Location	Habitat	A-S
Bathynerita naticoidea	FJ977768	FJ977721	Gulf of Mexico	Marine	1.
Clithon chlorostoma	EU732363	EU732200	South of Oku, Okinawa, Japan	Estuarine	2.
Clithon oualaniensis	EU732364	EU732201	Rowes Bay, Queensland, Australia	Estuarine	2.
Clithon corona	EU732199	EU732362	Suva, Viti Levu, Fiji	Freshwater	2.
Nerita funiculata	EU732245	EU732082	Esmeraldas Province, Ecuador	Marine	2.
Nerita scabricosta	EU732307	EU732144	Punta Conejo, México	Marine	2.
Nerita vitiensis	EU732357	EU732195	Koumac, New Caledonia	Marine	2.
Nerita undulata	EU732349	EU732187	Suva, Viti Levu, Fiji	Marine	2.
Nerita tristis	EU732323	EU732161	South of Oku, Okinawa, Japan	Marine	2.
Nerita textilis	EU732322	EU732159	KwaZulu-Natal, South Africa	Marine	2.
Nerita spengleriana	EU732315	EU732153	Huon Peninsula, Papua New Guinea	Marine	2.
Nerita reticulata	EU732312	EU732149	Doljo, Bohol, Filipinas	Marine	2.
Nerita sanguinolenta	EU732305	EU732143	Port Safâga, Egypt	Marine	2.
Nerita senegalensis	EU732310	EU732147	N'Gor, Senegal	Marine	2.
Nerita quadricolor	EU732304	EU732141	Sharm el-Nâga, Egypt	Marine	2.
Nerita polita	EU732301	EU732139	Phuket Island, Thailand	Marine	2.
Nerita plicata	EU732296	EU732133	Sabang, Mindoro, Philippines	Marine	2.
Nerita planospira	EU732292	EU732129	South of Poindimie, New Caledonia	Marine	2.
Nerita picea	EU732290	EU732127	Hawaii Island, Hawaii, USA	Marine	2.
Nerita patula	EU732286	EU732123	Balite, Mindoro, Philippines	Marine	2.
Nerita orbignyana	EU732284	EU732121	el-Qalawi, Egypt	Marine	2.
Nerita olivaria	EU732282	EU732119	Gorontalo, Sulawesi, Indonesia	Marine	2.
Nerita ocellata	EU732280	EU732117	South of Oku, Okinawa, Japan	Marine	2.
Nerita morio	EU732278	EU732115	Anakena, Easter Island	Marine	2.
Nerita melanotragus	EU732276	EU732113	Victoria Point, Moreton Bay, Queensland, Australia	Marine	2.
Nerita maxima	EU732274	EU732111	Huon Peninsula, Papua New Guinea	Marine	2.
Nerita magdalenae	EU732272	EU732109	Souillac, Mauritius	Marine	2.
Nerita longii	EU732270	EU732107	Haramel, Muscat, Oman	Marine	2.

Continued Table 2.

Species	# Acc. GenBank CO1	# Acc. GenBank 16S	Location	Habitat	A-S
Nerita litterata	EU732267	EU732104	Zanzibar Island, Tanzania	Marine	2.
Nerita japonica	EU732262	EU732099	Dol-San-Do, Yeo-Su, South Korea	Marine	2.
Nerita insculpta	EU732258	EU732095	Kusa Beach, Lombok, Indonesia	Marine	2.
Nerita incerta	EU732256	EU732093	Rakata Kecil, Krakatau, Indonesia	Marine	2.
Nerita histrio	EU732254	EU732091	Railey East, Krabi, Thailand	Marine	2.
Nerita helicinoides	EU732089	EU732252	South of Cape Hedo, Okinawa, Japan	Marine	2.
Nerita guamensis	EU732250	EU732087	Fadian Point, Guam	Marine	2.
Nerita grossa	EU732248	EU732085	Huon Peninsula, Papua New Guinea	Marine	2.
Nerita filosa	EU732242	EU732079	Natadola, Viti Levu, Fiji	Marine	2.
Nerita exuvia	EU732236	EU732073	Pantai Kulambu, Sabah, Malaysia	Marine	2.
Nerita erythrostoma	EU732234	EU732071	Mackay, Queensland, Australia	Marine	2.
Nerita costata	EU732232	EU732069	Cape Gaya, Sabah, Malaysia	Marine	2.
Nerita chamaeleon	EU732230	EU732067	East Coast Parkway, Singapore	Marine	2.
Nerita balteata	EU732226	EU732063	Mackay, Queensland, Australia	Marine	2.
Nerita aterrima	EU732222	EU732059	Cap La Houssaye, Reunion Island	Marine	2.
Nerita argus	EU732220	EU732057	Leone, American Samoa	Marine	2.
Nerita antiquata	EU732216	EU732053	Cape Gaya, Sabah, Malaysia	Marine	2.
Nerita yoldii	EU732359	EU732196	Lung Kwu Tan, Hong Kong	Marine	2.
Nerita umlaasiana	EU732325	EU732162	Mission Rocks, KwaZulu-Natal, South Africa	Marine	2.
Nerita atramentosa	EU732223	EU732060	Tasmania, Australia	Marine	2.
Neritina canalis	AY771270	AY771225	Moorea, French Polynesia	Freshwater	3.
Neritina turrita	AY771273	AY771227	Upolu, Samoa	Freshwater	3.
Neritina oweniana	EU732365	EU732202	Ada, Ghana	Freshwater	2.
Neritina rubricata	EU732369	EU732206	Ada, Ghana	Freshwater	2.
Puperita pupa	FJ977767	FJ977719	Gulf of Mexico (Caribbean)	Marine	1.
Septaria porcellana	AY771274	AY771228	Moorea, French Polinia	Freshwater	3.
Septaria sanguisuga	AY771275	AY771229	Tutuila, Samoa	Freshwater	3.
Theodoxus fluviatilis	AY765306	AY771240	Erstein, France	Freshwater	3.
Theodoxus baeticus	AY771277	AY771234	Quart, Spain	Freshwater	3.
Theodoxus meridionalis	AY771292	AY771253	Sortino, Sicily, Italy	Freshwater	3.

matrix = off; delay divergent cut-off = 30 %. The Clustal settings for the 16S RNA gene were: pairwise alignment parameters: gap open penalty = 15, extension penalty = 6,66; the multiple alignment parameters were: gap opening penalty = 15, extension penalty = 6,66; DNA weight matrix = IUB; transition weight = 0,5; negative matrix = off; delay divergent cut-off = 30 %.

Sequence Characterization

We analyzed the degree of saturation for the COI gene using the software DAMBE v. 5.3.0 (Xia and Xie, 2001). The percentage of A, T, C and G, together with the percentage of A + T and G + C for each region was calculated using MEGA (v.5) (Tamura *et al.*, 2011). We also calculated the number of synonymous vs nonsynonymous substitutions for the COI gene (Nei and Gojoborit, 1986) using the model Nei-Gojobori (Jukes-Cantor). The variance was estimated by the method of bootstraps using 1000 replicates in MEGA (v.5).

Phylogenetic Analysis

For phylogenetic analyses we used three matrices: 1) 16S gene; 2) COI gene; 3) Concatenated COI and 16S genes. Phylogenetic analyses were performed using Neighbor-joining (NJ), maximum parsimony (MP) and Bayesian inference methods (BI). The NJ and MP analyses were conducted in PAUP * version 4.0b10 (Swofford, 2002). Non-parametric bootstrapping was performed using a full heuristic search with 1000 replicates.

Bayesian inference analyses were conducted using Mr.Bayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The analysis model was chosen using MrModelTest (Nylander, 2004), with the AIC criterion. The GTR + G + I model was selected for both genes. For each analysis, four chains (three heated, one cold) were run simultaneously for the Monte Carlo Markov Chain. Two independent runs of 15 x 106 generations were performed, with trees sampled every 1000 generations. Each run started from a random tree. Asymptotic convergence to the posterior probability distribution was assessed by examining the plot of generation against the likelihood scores and confirmed using the sump command in software Mr. Bayes. Trees sampled prior to convergence were discarded before construction of the majority rule consensus tree. The percentage of sampled trees recovering a particular clade was used as a measure of that clades posterior probability (Huelsenbeck and Ronquist, 2001).

Although initial analyses using *Haliotis rubra* (Vetigastropoda: Haliotidae) and *Lophiotoma cerithiformis* (Caenogastropoda: Turridae) as outgroups were run, in these cases the internal groups clustered with the outgroup and several inconsistencies in the tree were obtained, consequently we decided to use the species *Bathynerita naticoidea* as outgroup. *B. naticoidea* is endemic to the Gulf of Mexico and lives in water depths from 400 m to 2100 m (Zande and Carney, 2001). This species is currently included in the family Neritidae, but it is probably more related to the family Phenacolepadidae, according to evidence from anatomical studies and embryology (Holthuis, 1995; Kano, 2006; Kano *et al.*, 2002).

Additionally, for the COI and the concatenated analyses, a BI analysis was performed separating each codon position (1st, 2nd, 3rd) of the COI gene as a partition. The model for each partition was calculated using MrModeltest 2.3, the model assigned to the first position was GTR + G, to the second position was F81 and to the third position was GTR + G. Substitution models and rates of substitution were allowed to vary among the parameters (unlink command and ratepr = variable).

Habitat Evolution

We manually mapped the habitat information for each species onto the consensus tree, assigning a different color to each habitat.

RESULTS

Sequence Characterization

After the exclusion of regions of questionable alignment, the concatenated dataset consisted of 1281 characters (722 bp for COI and 559 bp for 16S). For COI, the average frequency of each base was 38.8 % T, 17.5 % C, 22 % A, and 21.7 % G, and the percentage of A+T and G+C was 60.8 % and 39.2 % respectively. For 16S, the average frequency of each base was 32.5 % T, 19.6 % C, 29.3 % A, and 18.5 % G, and the percentage of A+T and G+C was 61.9 % and 38.1 % respectively. The number of synonymous substitutions per site for the COI region corresponded to 1,2 ± 0.08 (dS ± SE) and the number of nonsynonymous substitutions per site was 0.06 ± 0.006 (dN ± SE). The dN / dS ratio that defines the type of evolution was equal to 0.054, indicating a negative selection pressure acting on this gene (Pybus and Shapiro, 2009). Some degree of saturation was found for the third codon position of the COI region, I_{ss} 1.9385 > 0.8011 $I_{ss.c}$, but the position was included in all the analyses because we considered that this site presents valuable phylogenetic information that may not be detected with the first and second codon positions alone (Källersjö *et al.*, 1999; Frey and Vermeij, 2008; Xia, 2009).

Phylogenetic Analysis

Phylogenetic reconstructions of the COI region using Neighbor-Joining (NJ), Maximum Parsimony (MP), and Bayesian (BI) analyses produced similar topologies.

The result of the Bayesian analysis for COI (not shown) included a highly supported monophyletic group consisting of species of the genus *Nerita*; however, inter-node lengths within the genus were very short and in some groups it was not easy to see their internal relationships. The species of the genera *Neritina*, *Septaria*, *Clithon*, and *Puperita* formed another monophyletic group, in which *Neritina* virginea presented the longest branch, indicating slightly higher nucleotide substitution rates for this species. The genus *Theodoxus* formed a monophyletic group separate from the other groups. Although the MP analysis produced a tree with similar topology, the branches were longer and there was better resolution.

The analysis using the 16S region showed a divergent topology. It showed two groups, one group contained the species of the genus *Nerita* but with the inclusion of some species of the genus *Neritina*. In this case, *Nerita* was not monophyletic. The species of the genera *Septaria*, *Theodoxus*, *Clithon*, and *Puperita* appeared as a single group, but without a clear divergence and low support values on its nodes (not shown).

The consensus phylogenetic reconstructions (COI +16S) using BI formed three groups (Fig. 2a), a monophyletic group formed by the species of the genus *Theodoxus*, another monophyletic group formed by the species of the genus *Nerita* and another group with the remaining genera. The monophyly of the genus *Nerita* and the genus *Theodoxus* was supported with values of 1.00 and 0.89, respectively (Fig. 2a).

In the consensus analyses using MP and NJ, two groups were resolved. A monophyletic group consisting of species of the genus *Nerita*, with a high bootstrap support of 96 %, and another group with species of the genera *Neritina*, *Puperita*, *Theodoxus*, *Septaria*, and *Clithon* with a bootstrap support of 56 % (Fig. 2b). The topology of the MP tree showed longer branches in comparison to the Bayesian analysis, giving better structure to the tree.

The partitioned analysis of the consensus dataset (COI 1st + COI 2nd + COI 3rd + 16S) gave the same topology as the

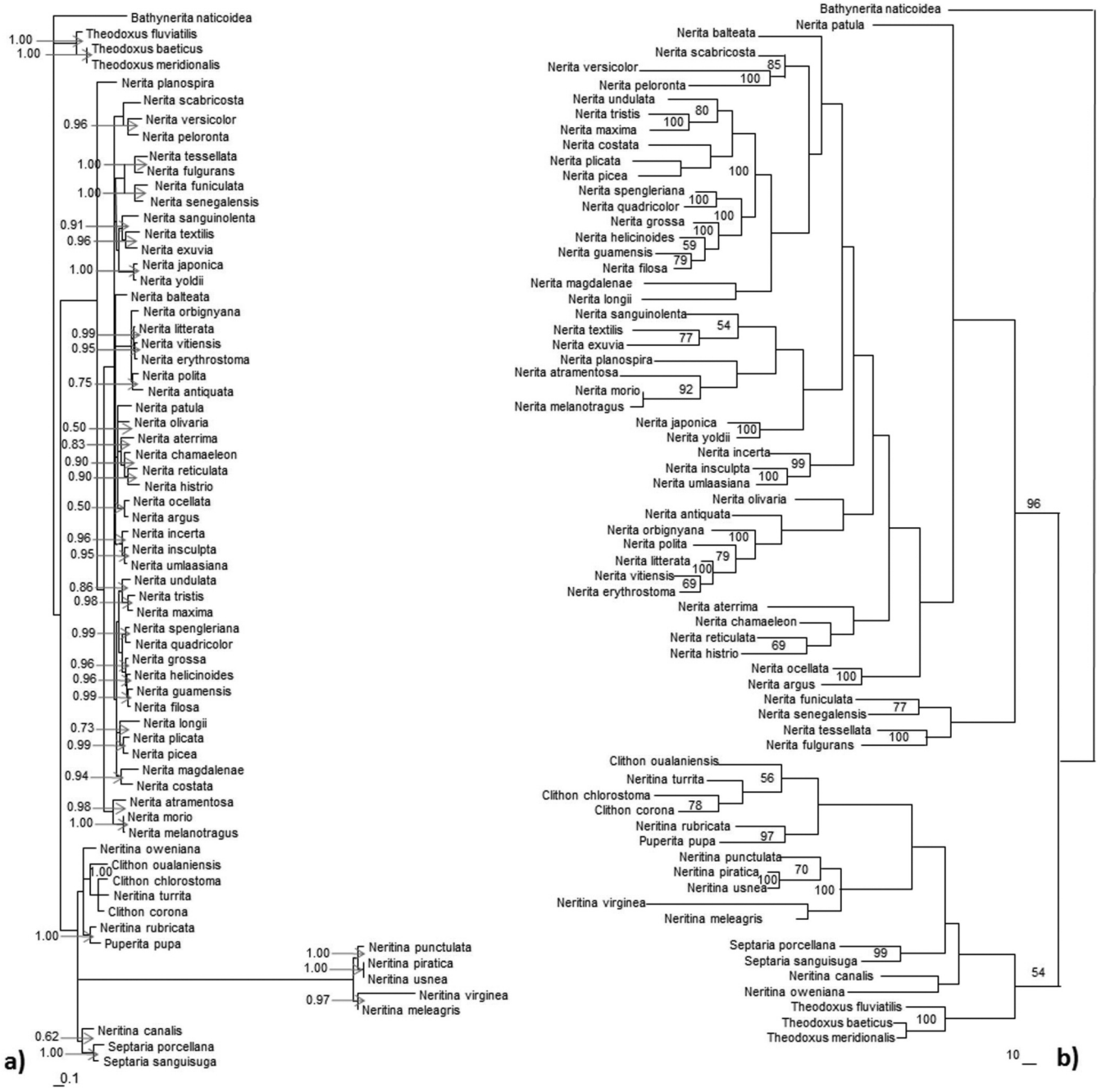

Figure 2. Phylogenetic tree of the species of the family Neritidae. a) Bayesian consensus analysis of the COI + 16S rRNA genes using the GTR + I + G model of evolution; the numbers correspond to posterior probability values. b) Maximum parsimony consensus analysis of the COI + 16S rRNA genes; the numbers on the branches correspond to bootstrap values (length = 3876, CI = 0.2745, RI = 0.6185). The scales correspond to the number of substitutions per site.

non partitioned analysis, however, the analysis without partitions showed higher support values on the nodes.

Mapping Habitat Information

The consensus tree obtained using the parsimony method was used to map the habitat information of each species (Fig. 3). Based on the tree topology, this analysis supports the hypothesis that the family Neritidae originated in marine environments.

DISCUSSION

Phylogeny Of The Family Neritidae

Six percent of the world's known species of *Nerita* occur in the Colombian Caribbean (Frey and Vermeij, 2008; Frey, 2010b), as do 0.5 % of the species of *Neritina* that inhabit freshwater and estuarine habitats (Kano *et al.*, 2006). In addition, one species of each of the genera *Puperita* and

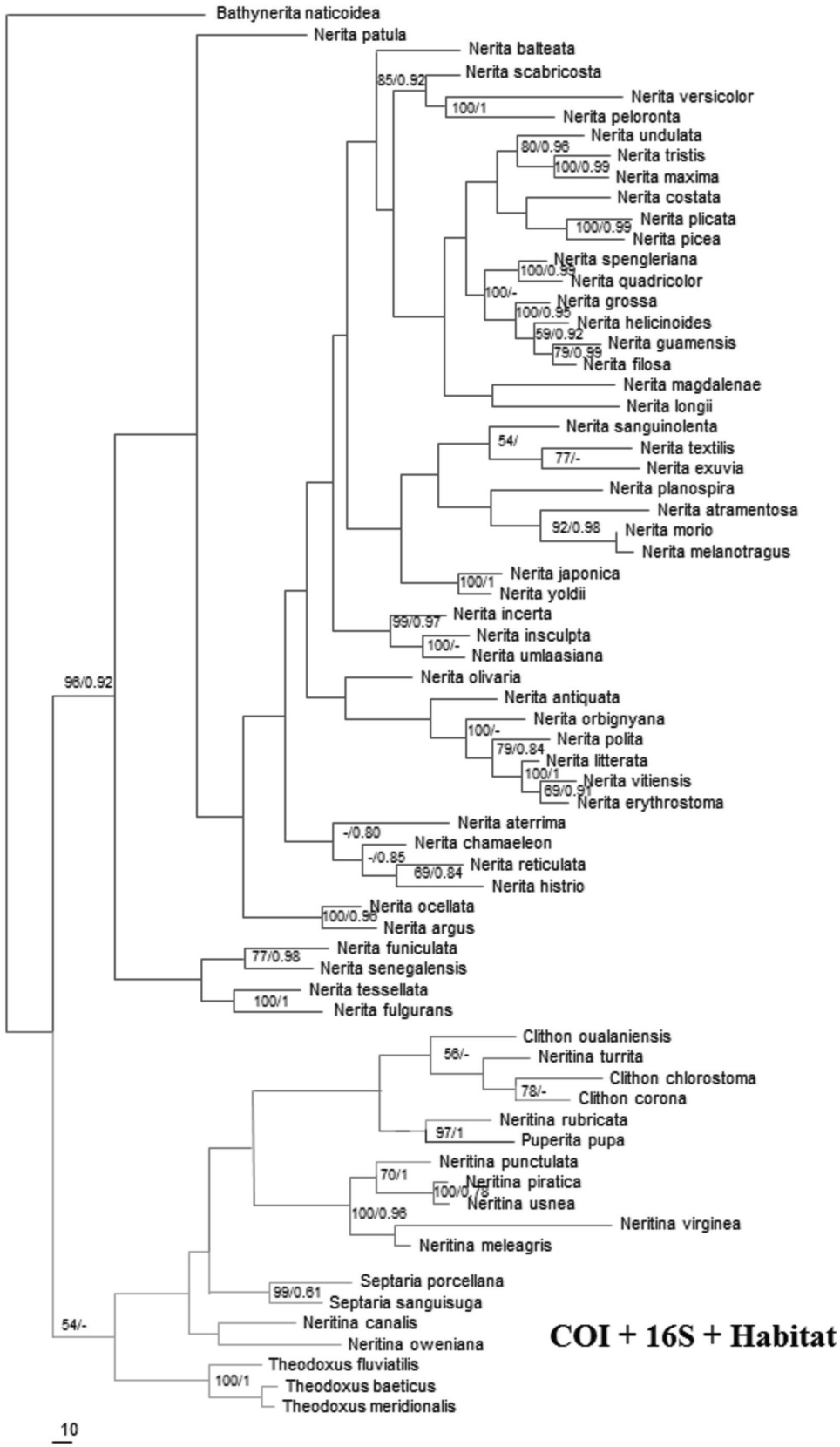

Figure 3. Consensus tree (COI +16 S) of the Family Neritidae, using the topology of the parsimony analysis. The numbers correspond to MP bootstrap values / Bayesian posterior probability values. The scale is the number of substitutions per site. The colors represent the habitat of each species and the inferred habitat of deeper branches: blue = marine, green = estuarine, orange =freshwater.

Smaragdia, occur in the Colombian Caribbean. As of July 2012, 44 species of the genus *Nerita* were represented in GenBank, which corresponds to approximately 87 % of the species reported in the world. In contrast, only 13 species belonging to the genera *Neritina*, *Puperita*, *Septaria*, and *Clithon* were reported in GenBank, representing only 7.5 % of the known species of these groups. Most of these sequences were generated through studies in Europe, Japan and the coasts of North America (Bunje and Lindberg, 2007; Hurtado *et al.*, 2007; Frey and Vermeij, 2008; Kano, 2009; Aktipis and Giribet, 2010). No species of Neritidae known from the Colombian Caribbean were previously represented in Gen Bank. Further, only one complete mitochondrial genome is available for the Neritimorpha, that of the Australasian *Nerita melanotragus* (Castro and Colgan, 2010). Thus, additional sampling with expanded taxonomic, as well as geographic coverage is needed to further resolve the phylogeny of the family. Specifically, additional studies including poorly represented (*Neritina*, *Puperita*, *Clithon*, *Septaria*), or unrepresented (i.e., *Fluvinerita*, *Neripteron*, *Nereina*, *Clypeolum*, *Neritodryas*) genera are needed. In this study, we sequenced nine species of neritid snails belonging to the genera *Nerita* and *Neritina* from the Colombian Caribbean, and used them, together with the sequences available in GenBank to perform an integrated analysis of the family Neritidae.

Our phylogenetic reconstructions revealed a similar topology for COI both with parsimony or BI, separating three groups, one consisting of the monophyletic genus *Nerita*, another with the species of the genus *Theodoxus*, and a third group including all the other genera. The analyses showed high support on the basal nodes, confirming that the COI region is a good marker for evaluating and resolving hypotheses about the evolution of the group (Remigio and Hebert, 2003; Frey and Vermeij, 2008). The 16S gene, on the other hand, is considered a good phylogenetic marker in terrestrial mollusks (Klussmann-Kolb *et al.*, 2008), however, the trees produced in our analyses were not consistent, giving different topologies for the MP, NJ and BI analyses. This finding corroborates the results of Frey and Vermeij (2008), who considered the 16S gene as unstable and less useful for reconstructing phylogeny. In our consensus analysis (COI +16S) of the family Neritidae, the genus *Nerita* is monophyletic. This finding is consistent with the results obtained by Frey and Vermeij (2008) who evaluated the molecular phylogeny and biogeography of *Nerita*. In our analysis the species of the genera *Neritina*, *Puperita*, *Septaria*, *Theodoxus*, and *Clithon* formed a monophyletic group by the parsimony method, however the genus *Theodoxus* was recovered as a third independent monophyletic group using BI, causing uncertainty about the phylogenetic position of this group within the family, since in both cases the support values were high.

Holthuis (1995), proposed a phylogenetic tree from an anatomical study of the genera and subgenera of the families Neritidae, Phenacolepadidae, and Septariidae, based on 57 morphological characters. She considered each genus within the Neritidae as a monophyletic group, an assumption not consistent with the results obtained in the present study, which is the first attempt at integrating all the molecular information available. We strongly recommend the inclusion of a larger number of genera and species, as well as other molecular markers, in studies to further resolve the systematics of the group.

Regarding the species collected in the Colombian Caribbean, *Nerita versicolor* and *Nerita peloronta* formed a clade that is strongly supported by posterior probabilities and bootstrap values, this clade was always associated with the species *Nerita scabricosta*. This finding is consistent with what was reported by Frey and Vermeij (2008), who placed these species in the subgenus *Nerita sensu stricto*. *Nerita fulgurans* and *Nerita tessellata* also formed a well supported monophyletic group, which, along with *Nerita senegalensis* and *Nerita funiculata*, represents the subgenus *Theliostyla* (Frey and Vermeij, 2008; Frey, 2010b). The species *Neritina punctulata*, *Neritina piratica*, *Neritina usnea*, *Neritina virginea*, and *Neritina meleagris*, appeared as a monophyletic group in the consensus analysis, but the individual analysis of the COI gene recovered *N. punctulata* + *Puperita pupa* and *N. rubricata* + (*N. virginea* + *N. meleagris*) as monophyletic groups, and the species *N. piratica* and *N. usnea* formed another well supported monophyletic group with species of the genera *Clithon* and *Septaria*.

It is important to highlight the close relationship of the species *N. piratica* and *N. usnea* that was recovered in all the analyses, which is consistent with Russell's (1941) suggestion that they should be considered a single species (based on conchological characteristics). Since no anatomical or molecular analyses have been used to resolve their taxonomic status, these taxa have continued to be considered as separate species (Yidi and Sarmiento, 2010). The sequences generated in this study for *N. piratica* and *N. usnea*, showed no differences in the COI region, and only a three base pair difference in the 16S region between these two species. In contrast, comparison of these sequences with those of *N. virginea*, showed 19 bp and 123 bp differences for the COI and the 16S regions respectively; *N. versicolor* and *N. peloronta* (another very closely related species pair) presented 460 and 257 bp different in the COI and 16S genes, respectively. We conclude that there is little difference at the molecular level between *N. piratica* and *N.usnea*, and question their classification as different species. We suggest a more detailed population study and additional examination both at the anatomical and the molecular levels to further assess the status of these taxa.

Evolution of the Family in Relation to Habitat

Adaptive radiation is a response to natural selection and ecological opportunity that involves diversification of species with accompanying adaptations (Glor, 2010). Neritimorpha is a superorder that has undergone significant adaptive radiation, and has an extensive fossil record. It includes

representatives that have invaded from marine environments to terrestrial habitats, mainly during the Carboniferous period. Families that currently occur in terrestrial environments include Hydrocenidae, Helicinidae, Proserpinidae, and Ceresidae (Thompson, 1980; Kano *et al.*, 2002; Kano *et al.*, 2006), whereas the family Neritidae includes extant representatives in marine, estuarine, and freshwater environments (Kano *et al.*, 2002; Kano *et al.*, 2006).

Mapping habitat on the phylogenetic reconstruction of the species of the family Neritidae (Fig. 3), makes evident the relationship of the species of the family with marine environments, and strongly suggests evolution from marine to freshwater environments, as proposed by Kano *et al.*, (2006). The genus *Nerita*, fully occurring in marine habitats, is recovered as a monophyletic group, whereas the species with freshwater and estuarine habitats formed a separate group (although *Puperita pupa*, which is marine, was recovered within this group). Holthuis (1995) proposed, by an anatomical analysis of the species of the family Neritidae, a parsimonious reconstruction indicating that at least 12 changes have occurred during the evolution among marine, freshwater, and estuarine environments. According to this author, multiple invasions from marine to freshwater environments have occurred. Multiple invasions have also been hypothesized for the genus *Septaria* entering freshwater streams in tropical Pacific islands (Ponder 1998), and in the radiation of the genus *Theodoxus* in the river systems of Europe and Central Asia (Bunje and Lindberg, 2007; Bunje, 2007). Some freshwater species of the genus *Neritina* still have a larval stage in estuarine or marine environments before returning to freshwater streams and rivers (Blanco and Scatena, 2006; Kano, 2009; Gorbach *et al.*, 2012), further supporting the hypothesis that the family has its origins in the sea.

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