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Molecular phylogenetics reveals differential divergence of coastal snails separated by the Isthmus of Panama

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ABSTRACT

We used 20 species of coastal marine snails in the genus *Cerithidea* and *Cerithium* collected along the Pacific and Atlantic coasts of Central America to investigate the role of the rise of the Isthmus of Panama in the speciation of this group. Of particular interest was the identification of geminate species pairs presumably established by the disruption of gene flow across the isthmian barrier. Hypotheses of phylogenetic relationships were based on approximately 2.4 Kb of the mitochondrial cytochrome oxidase *c* subunit I gene, 16S ribosomal RNA gene and the nuclear 28S ribosomal gene. We identified four putative geminate species pairs out of the 20 species evaluated, but the level of sequence divergence among the pairs differed more than two-fold. A geminate pair, in which both species live in the high intertidal of mangrove habitats, exhibited less sequence divergence compared to other pairs occupying lower intertidal and subtidal habitats. Mangrove dwelling species were probably the last to be separated by the final closure of the Central American Seaway, and thus their divergence times correspond most accurately to the completion of the Isthmus.

1. Introduction

Reliable estimates of divergence times are crucial for understanding evolutionary histories. While molecular clocks are often based on fossil records, gaps in the records and uncertainty interpretations can limit accurate calibration. Alternatively, well-dated biogeographic barriers such as the Isthmus of Panama can provide another way to estimate divergence times. The Isthmus of Panama rose over 16–3 million years ago, gradually separating the Pacific and Atlantic ocean basins (Coates and Obando, 1996), and eventually creating a barrier to gene flow between marine organisms on either side. A large number of closely related species, so-called geminate species pairs (Jordan, 1908), occur along both coasts of Central America (Mayr, 1954; Lessios, 1998). These "geminate species" provide opportunities to evaluate factors driving biological diversity (Vermeij, 1993), because the time since the isolation between the species can be estimated using the geological record. The final closure of Central American Seaway provides the basis for inferring rates of molecular evolution (Bermingham and Lessios, 1993; Knowlton et al., 1993; Collins, 1996; Donaldson and Wilson, 1999; McCartney et al., 2000; Lessios et al., 2001; Bellwood et al., 2004; Lee and Ó Foighil, 2005), and in turn can be used to estimate divergence times.

Nonetheless, there are two basic assumptions implicit in the geminate species concept. First, geminate species are by definition sister taxa, and second, the rise of the Isthmus of Panama is hypothesized to be the cause of the separation of an ancestral species into daughter species on either side of the Isthmus. The advent of molecular genetics has established probable cases of misidentification of geminate species based on morphology as deduced from molecular phylogenies (see Lessios, 2008). Further, establishing the time of separation between geminates is fraught with problems, as the emergence of the isthmian landmass was a prolonged geological process (Coates and Obando, 1996; Lessios, 2008). Recent molecular evidence suggests that all geminates were not simultaneously separated by the emerging Isthmus (Knowlton and Weigt, 1998; Marko, 2002). As a case in point, Knowlton et al. (1993) and Knowlton and Weigt (1998) demonstrate a positive association between habitat depth and evolutionary distances of geminate shrimp species in the genus *Alpheus*. Geminate shrimp species pairs occupying near-shore mangrove habitats exhibited genetic distances that were less than their deep water congeners.

Houbrick (1974) and Vermeij (1978) identified possible molluscan geminate species pairs on either side of the Isthmus of Panama, including Cerithioidean snails in the genus *Cerithidea* and *Cerithium*. These snails inhabit shallow tropical and warm temperate seas in the Indo and eastern Pacific, the western and eastern

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Atlantic (and Mediterranean for *Cerithium*). There are about twenty living species in *Cerithidea* (Reid et al., 2008) and about forty living species in *Cerithium* in the world (Houbrick, 1974), although many of these are in critical need of taxonomic revision. Eight species of *Cerithidea* and 16 species of *Cerithium* are reported from the Americas (Bequaert, 1942; Keen, 1971; Houbrick, 1974; Reid et al., 2008). While collecting and identifying all of the *Cerithidea* species is relatively straightforward, taxonomic uncertainties and limited distributions make this more difficult for *Cerithium*. Of the 16 putative *Cerithium* species in the Pacific, four (*C. browni*, *C. maculosum*, *C. gallapaginis* and *C. mediolaeve*) are morphologically similar and often considered synonymous or subspecies of others (Keen, 1971). Despite taxonomic complexities, based on morphological similarity, *Cerithium uncinatum* and *C. atratum*; *C. menkei* and *C. lutosum*; *C. littratum* and *C. stercusmuscarum*; *Cerithidea montagnei* and *C. costata* were identified as potential geminate species pairs (Houbrick, 1974; Vermeij, 1978). However, since morphological similarities may limit the ability to accurately identify geminate species pairs (Lessios, 1998), it is useful to evaluate the validity of these pairs using independent criteria. Most Central American species within these genera are associated with near-shore habitats including mangroves (Keen, 1971; Houbrick, 1974; Reid et al., 2008) and thus provide an ideal system to test rates of molecular evolution across the Isthmus. Similar to mangrove shrimps (Knowlton and Weigt, 1998), mangrove snails likely maintained gene flow across the emerging isthmus, until the separation of eastern Pacific and Caribbean mangroves habitats was completed. We evaluated genetic divergence of *Cerithium* and *Cerithidea* across the Isthmus of Panama to begin to test the hypothesis that the emergence of Isthmus simultaneously inhibited gene flow of these near-shore snail species.

2. Materials and methods

2.1. Sampling and sequencing

We collected all eight *Cerithidea* species and nearly all *Cerithium* species (except the four species described above) from both coasts of the tropical Americas (Table 1). Snails were either frozen or preserved in 95% EtOH. All samples were stored at −20 °C in the laboratory for molecular analyses. We isolated DNA using a modified procedure from Doyle and Doyle (1987). A small piece of tissue from the foot of each snail was homogenized in a solution of 300 mL 2× CTAB and 10 mg mL^{-1} proteinase K, and incubated at 60 °C for approximately 1 h, extracted once with phenol/chloroform (v:v, 1:1) and precipitated with 2 vol of ethanol. The DNA pellets were briefly washed in 75% ethanol, air-dried for approximately 30 min and dissolved in 50 ml of H_2O.

We analyzed mitochondrial DNA encoding the cytochrome oxidase *c* subunit I (COI) gene, 16S ribosomal RNA gene and the nuclear 28S ribosomal RNA gene. While the 28S gene is generally conservative and is not ideal for genetic resolution at the species level, given the absence of other more appropriate nuclear markers for these taxa, we included it to evaluate potential hybridizations between these snails. As primer pairs, we used COI-bf (Miura et al., 2006a) and COI-6 (Shimayama et al., 1990) for amplification of the COI gene, 16Sar and 16Sbr (Kessing et al., 1989) for the 16S gene, and D1F and D6 (Park and Ó Foighil, 2000) for the 28S gene. For the COI gene, PCR were run for 35 cycles under the following conditions: denaturing at 94 °C for 60 s, annealing at 45 °C for 60 s and extension at 72 °C for 90 s. The 35 cycles were preceded by an initial denaturing at 94 °C for 1 min. followed by a final extension of 72 °C for 7 min. For the 16S gene, the same PCR conditions were used but annealing temperature was 50 °C. A touchdown protocol was used for the 28S gene amplification. After 1 min. of denaturation at 95 °C, an initial annealing temperature of 65 °C was decreased by 2 °C/cycle until the final annealing temperature of 50 °C was reached and subsequently maintained for an additional 30 cycles under the following conditions: denaturing at 95 °C for 30 s, annealing at 50 °C for 40 s, extension at 72 °C for 90 s and a final extension of 72 °C for 7 min. The PCR products of the samples were purified and sequenced using automated sequencer (ABI 3130xl). Sequences analyzed in this study were deposited in GenBank (Accession Nos. GQ273758–GQ273892, GU246113–GU246120).

2.2. Estimation of phylogenetic relationships and genetic parameters

Sequences were aligned by ClustalW, implemented in BioEdit (Hall, 1999) for further phylogenetic analyses. All insertions and deletions (indels) were removed from the alignment before phylogenetic analyses. Phylogenetic trees were constructed using Maximum likelihood (ML) algorithms and Bayesian inference (BI). We selected the best evolutionary models for each gene and combined dataset using Modelgenerator (Keane et al., 2006) for ML analyses and MrModeltest (Nylander, 2004) for BI analyses (Table 2). For the

Table 1
Snails used for molecular analyses with localities of samples and numbers of the analyzed individuals for COI, 16S and 28S genes.

Genus	Species	Sampling Location	Ocean	CO1	16S	28S
Cerithideau	*C. californica* (Haldeman, 1840)	Santa Barbara, California, USA	Pacific	5	3	3
	C. mazatlanica (Carpenter, 1857)	Bique, Panama	Pacific	5	3	3
	C. valida (Adams, 1852)	Rio Venado, Panama	Pacific	6	4	4
	C. pliculosa (Menke, 1829)	Colon, Panama	Atlantic	5	3	3
	C. pulchra (Adams, 1852)	Gulfo de Montijo, Panama	Pacific	5	3	3
	C. montagnei (Orbigny, 1839)	Rio Venado, Panama	Pacific	4	3	2
	C. scalariformis (Say, 1825)	Hillsborough, Florida, USA	Atlantic	4	3	3
	C. costata (da Costa, 1778)	Yal-ku, Quintana Roo, Mexico	Atlantic	5	3	1
Cerithium	*C. atratum* (Born, 1778)	Colon, Panama	Atlantic	5	3	3
	C. guinaicum (Philippi, 1849)	Carrie Bow Cay, Belize	Atlantic	3	2	3
	C. uncinatum (Gmelin, 1791)	Las Perlas, Panama	Pacific	3	3	3
	C. nicaraguense (Pilsbry and Lowe, 1932)	Isla Coiba, Panama	Pacific	4	2	2
	C. adustum (Kiener, 1841)	Isla Taboga, Panama	Pacific	4	2	2
	C. stercusmuscarum (Valenciennes, 1833)	Bique, Panama	Pacific	5	3	2
	C. menkei (Carpenter, 1857)	Rio Mar, Panama	Pacific	3	3	3
	C. muscarum (Say, 1832)	Fort Pierce, Florida, USA	Atlantic	5	2	2
	C. lutosum (Menke, 1828)	Colon, Panama	Atlantic	4	3	3
	C. eburneum (Bruguière, 1792)	Corn Island, Nicaragua	Atlantic	5	3	1
	C. litteratum (Born, 1778)	Colon, Panama	Atlantic	4	3	3
	C. gemmatum Hinds, 1844	Las Perlas, Panama	Pacific	3	3	3

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Table 2
The best evolutionary models selected for the ML and BI analyses.

Group	Genes	ML	BI
Cerithidea	CO1	HKY + G	HKY + G
	16S	GTR + I + G	GTR + I + G
	28S	TrN + I	GTR + I
	Combined	GTR + I + G	–
Cerithium	CO1	TVM + I + G	GTR + I + G
	16S	GTR + I + G	GTR + I + G
	28S	GTR + I	GTR + I
	Combined	TIM + I + G	–
Geminates	Combined	TIM + I + G	–

combined dataset of three genes, concordance of the analyzed genes was evaluated with the partition homogeneity test implemented with PAUP* (Swofford, 2003). One thousand randomizations were conducted using a heuristic search with tree bisection-reconnection branch swapping (TBR) and the maximum number of trees was constrained to 10,000. The ML searches were run under the appropriate evolutionary models using PHYML (Guindon and Gascuel, 2003). Node robustness was assessed using non-parametric bootstrapping and 1000 replicates. *Cerithium nicaraguense*, *C. stercusmuscarum* and *C. gemmatum* were used as outgroups for the *Cerithidea* phylogeny. *Cerithidea mazatlanica*, *C. montagnei* and *C. costata* were used as outgroups for the *Cerithium* phylogeny. The BI analyses were conducted using MrBayes (Huelsenbeck and Ronquist, 2001). Each dataset was run for 3 million generations with a sample frequency of 100. The first 25% of trees were discarded such that only 22,501 trees were accepted. Since the software automatically analyzed the data in two independent runs, a total of 45,002 trees were analyzed to estimate phylogenetic relationships and posterior probability value of each clade. Convergence between the two runs was tested by examining the potential scale reduction factors. We used gene specific models for the combined dataset in the BI analyses. We examined the consistency of the evolutionary rate in the combined dataset in order to determine whether the application of a molecular clock was appropriate for geminate species pairs. For this, we selected potential geminate species pairs on the basis of phylogenetic analyses and used a tree-wide likelihood ratio test (Felsenstein, 1981). The best evolutionary model was selected using Modeltest (Posada and Crandall, 1998) and likelihood scores with and without a molecular clock were estimated using PAUP* (Swofford, 2003). Divergence times of geminate species pairs were estimated using BEAST 1.5.2 which employs Bayesian Markov chain Monte Carlo algorithm (Drummond and Rambaut, 2007). We used potential geminate species pairs and set each pair as monophyletic group. A molecular clock was calibrated using a pair of geminate species which was most likely separated by the final closure of the Isthmus. The Yule speciation model was used as a tree prior. Since consistencies in the rate of molecular evolution was not rejected in the combined dataset (see Section 3), a strict molecular clock was applied to estimate divergence times of geminate species pairs. The analysis was run for ten million generations sampled every one thousand steps and the first 1000 samples were discarded as burn-in. To check for convergence and to visualize the results we used Tracer 1.4.1 and FigTree 1.2.3 (Drummond and Rambaut, 2007).

3. Results

The phylogenies estimated by BI were consistent with the phylogeny generated by the ML algorithm. The COI gene data provided finer resolution at shallow nodes (Fig. 1). Most of species were resolved with high support. However, *Cerithidea californica*, *C. mazatlanica* and *C. valida* formed a single clade and some individuals shared identical haplotypes between these nominal species. Also, a few individuals of *C. valida* and *C. pliculosa* formed a single clade with high support values. Similarly, *Cerithium atratum* and *C. guinaicum* formed a single clade with high support values. Topology inferred by 16S dataset was similar to that inferred by COI, but the 16S tree support values were higher for the internal nodes (Fig. 2). Specifically, the 16S dataset supported the monophyly of Pacific *C. stermuscuscrum* and Atlantic *C. lutosum* with high support values (ML/BI = 94%/96%), however, this relation was not resolved by either the CO1 or the combined datasets. The 28S ribosomal RNA gene data generally provided fine resolution for deep nodes. However, since there were low levels of variation in the 28S gene, many species shared identical genotypes, and support values for the terminal nodes were low (Fig. 3). A single individual of *C. valida* which had a similar mitochondrial haplotype to *C. pliculosa* had an identical 28S genotype to *C. pulchra* (see *C. valida* 3 in Figs. 1A–3A). The partition homogeneity test indicated that these three genes could be combined ($P = 0.99$). Thus, we constructed a phylogenetic tree based on the combined 2.4 Kb dataset (Fig. 4). The combined dataset provided maximum support for all generic and species clades. The inferred phylogenetic tree demonstrated that Pacific *Cerithium uncinatum* and Atlantic *C. atratum* and *C. guinaicum* form a single clade, and that Pacific *Cerithidea californica*, *C. mazatlanica*, *C. valida* and Atlantic *C. pliculosa* form a single clade. These two clades exhibited high support values (90–100%). Further, the estimated topology suggested that Pacific *Cerithidea montagnei* and Atlantic *C. scalariformis* form a single clade with relatively high support values (76%/91%). Pacific *Cerithium stercusmuscarum* and Atlantic *C. lutosum* form a single clade but the support values are relatively low (51%/82%). Note that the combined phylogeny could be highly influenced by variations in mitochondrial genes since the 28 gene had limited phylogenetic signal in shallow nodes. Consistencies of the rate of molecular evolution was not rejected in the combined dataset (difference in $-\ln = 33.6$, $P = 0.26$). The estimated divergence times are shown in Table 3.

4. Discussion

4.1. Species complex

While the Pacific *C. californica*, *C. mazatlanica* and *C. valida* exhibit some morphological variation, they shared identical mitochondrial haplotypes and the same 28S genotypes (Figs. 1A and 3A). Similarly, the Atlantic *Cerithium atratum* and *C. guinaicum* shared identical mitochondrial haplotypes and nuclear genotypes. This suggests that either introgression among the species occurred, or that the snails are conspecific. However, if these snails represented different species, we would expect greater phylogenetic diversity and structure, generated by independent mutations in distinct reproductive groups, compared to what we observed. Thus, these nominal species may actually be single polymorphic species complexes.

Indeed, Keen (1971) suggests morphological similarities between *C. californica*, *C. mazatlanica* and *C. valida*. The main differences between these putative snail species are the sizes and shapes of the shells, both of which can vary within a single species across estuaries with different environments and biotic conditions. For example, parasitism is one factor which explains intraspecific variation in shell morphology of mud snails (e.g., Lafferty, 1993; Miura et al., 2006b). Our genetic results and observed morphological comparisons suggest that each of these nominal species are probably morphological variations within a single species. However, these species complexes are still open to taxonomic repartitioning

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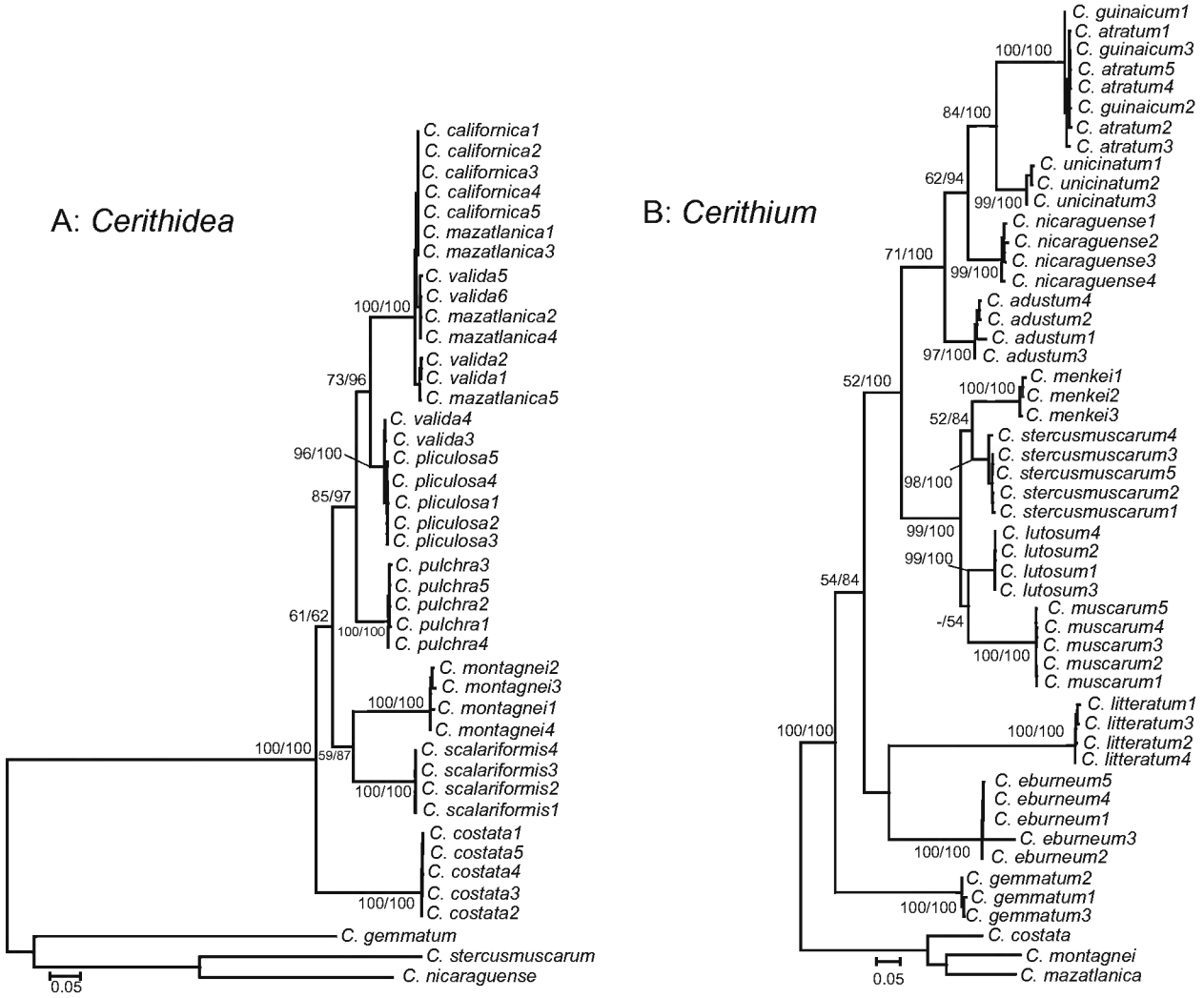

Fig. 1. Maximum likelihood trees based on the mitochondrial COI gene (878 bp) for (A) *Cerithidea* and (B) *Cerithium*. Numbers near nodes are the support values for the clade from the different analyses in order: ML/BI (values < 50% not shown).

pending identification of a parental clade for each nominal species or evidence of reproductive isolation.

4.2. Shells vs. molecules

We found several pairs of closely related snail species between the Pacific and Atlantic Oceans. Morphologically, *Cerithium uncinatum* from the Pacific and *C. atratum* from the Atlantic are very similar and are proposed to be geminate species (Houbrick, 1974; Vermeij, 1978). Our genetic data demonstrate that the *C. atratum* species complex (hereafter referred to as "*C. atratum*") and *C. uncinatum* indeed form a single clade with high support values (Figs. 1B–4B). *C. uncinatum* inhabits the subtidal zone and occurs on sand and coral reef rubble. *C. atratum* occurs in similar habitats and their ecological similarities are consistent with their close genetic relationship. Geminate species identifications based on morphology are, however, not always accurate. Pacific *Cerithium stercusmuscarum* and Atlantic *C. litteratum* are morphologically similar (Houbrick, 1974), but are genetically distantly related (Figs. 1B–4B). Similarly, Pacific *Cerithium menkei* and Atlantic *C. lutosum* are also proposed to be geminate species (Vermeij, 1978), but our molecular phylogenies provide no resolution for this (see Figs. 1A–4A). However, the close relationship between *C. stercusmuscarum* and *C. lutosum* are supported on the basis of the 16S phylogeny (Fig. 2B), suggesting they are geminate species. Note that we consider inference regarding this geminate species pairs as tentative since this relationship was unresolved based on the COI and combined dataset. *Cerithium stercusmuscarum* exhibits substantial variation in shell morphology and ornamentation, which may obscure the morphological similarity between the snails. *C. stercusmuscarum* and *C. lutosum* share habitat similarities – they are often both associated with mangroves where marine and freshwater mix, while most of *Cerithium* species in the Americas are fully marine. Although the Pacific *Cerithidea montagnei* and Atlantic *C. costata* are proposed to be geminate species (Vermeij, 1978), our data show that *C. costata* is a phylogenetically basal group in the American *Cerithidea*, and is not closely related to any other *Cerithidea* species (Figs. 1A–4A). However, the phylogenetic inference based on the combined dataset suggests that the Pacific *C. montagnei* and Atlantic *C. scalariformis* could be geminate species. Consistent with this, *C. montagnei* and *C. scalariformis* have several ecological similarities. Both species live in the upper intertidal within mangroves and unlike any other American *Cerithidea*, both consistently climb mangrove trees avoiding submersion at the high tide. Additionally, both snails are relatively fast growing and only develop a single terminal varix, whereas other *Cerithidea* are long-lived, relatively slow growing and often form several varices.

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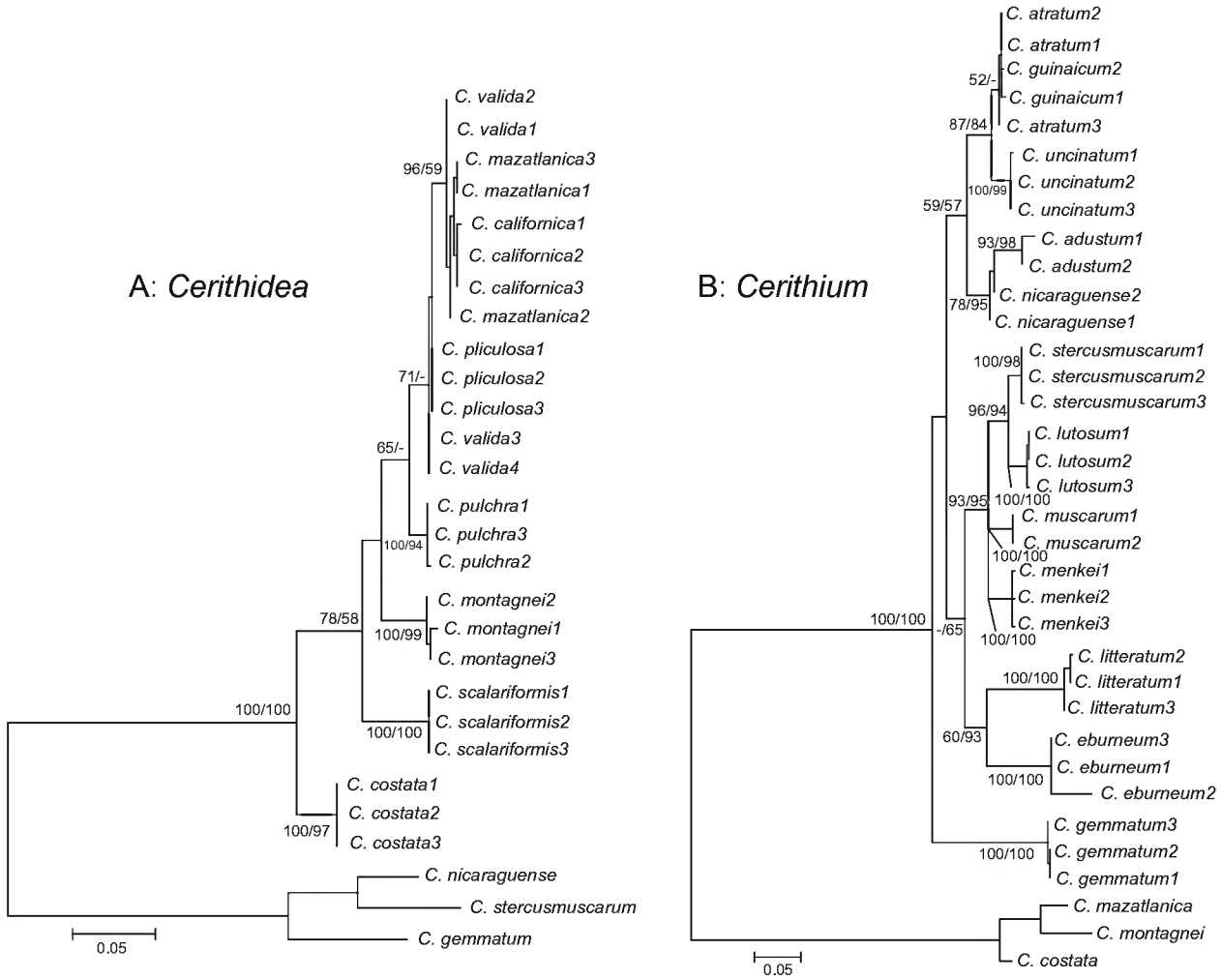

Fig. 2. Maximum likelihood trees based on the mitochondrial 16S gene (497 bp) for (A) *Cerithidea* and (B) *Cerithium*. Numbers near nodes are the support values for the clade from the different analyses in order: ML/BI (values < 50% not shown).

Our complete taxonomic sampling for *Cerithidea* and nearly complete sampling of *Cerithium* (see above) in the Americas, enables robust inferences regarding these geminate species pairs. In addition, we included additional sequences available from GenBank in our analyses to evaluate whether our inference of geminate species pairs would be influenced by adding the sequences of *Cerithidea* and *Cerithium* from other parts of the world. Our analyses suggest that *Cerithidea* and *Cerithium* species from other parts of the world are not closely related to the snails in the Americas (Supplementary Fig. 1A and B). However, phylogenetic resolution was generally low because GenBank only had a limited number of sequences available for congeneric snails and our dataset shared only a short consensus sequence with these. Future studies on congeneric snails from elsewhere in the world would provide the data needed for higher resolution of these relationships.

4.3. Cerithidea californica complex and C. pliculosa

The *C. californica* species complex (hereafter referred to as "*C. californica*") from the Pacific and *C. pliculosa* from the Atlantic formed a single genetic clade with high support values (90%/100%, Fig. 4A), suggesting they are geminate species. Again, these snails are ecologically similar, *C. californica* and *C. pliculosa* occur on sandy, muddy habitat in the upper shore of the mangroves and salt marshes (in the north). Interestingly, a mitochondrial haplotype group commonly observed in the Atlantic *C. pliculosa* was also found in the Pacific *C. californica* (see *C. valida* 3 and 4 in Figs. 1A and 2A). As we discuss below, geminate gastropods separated by the Isthmus exhibit at least 7% of sequence divergence in the COI gene and at least 0.6% of sequence divergence in the 16S gene (Lessios, 2008). However, sequence divergence between these snails is extremely low (0.8% in the COI gene and 0.2% in the 16S gene), suggesting that a potential dispersal event occurred between the Pacific and Atlantic after the final closure of the Isthmus and introgressive hybridization occurred recently between these two species. There are several examples which suggest that dispersal between the Pacific and Atlantic occurred after the Isthmus rose, by the breaching (Lessios et al., 2001) and by human activities (Roy and Sponer, 2002; Miglietta and Lessios, 2009). We are currently sampling more extensively across the Americas to fully evaluate this possibility and the potential pathways for recent dispersal events between the Pacific and Atlantic coasts.

4.4. Times of divergence between geminate snails

Geminate species pairs should exhibit similar evolutionary distances if the emergence of the Isthmus simultaneously isolated their populations. However, the average evolutionary distances of the four potential geminate species pairs in our study varied (Fig. 5). While this could be explained by different rates of

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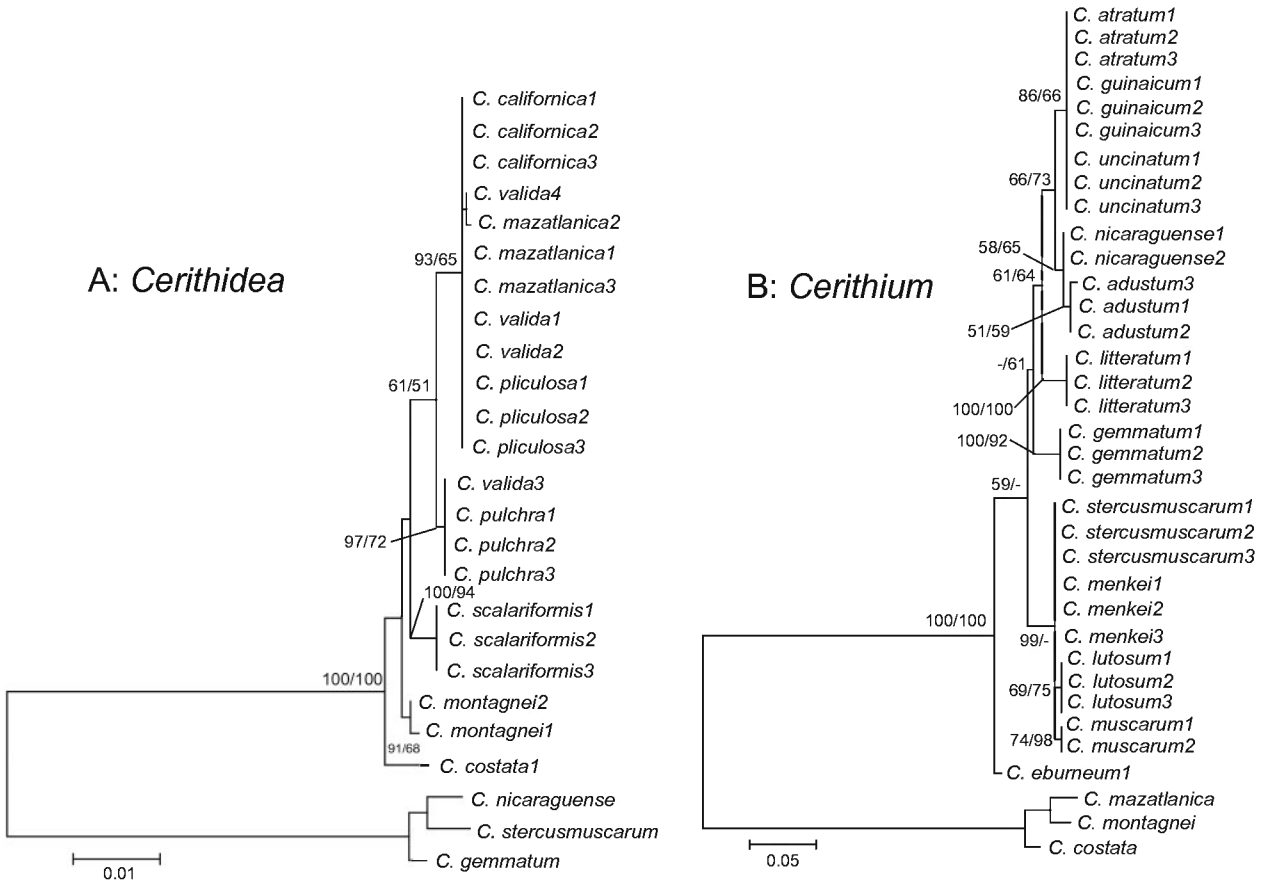

Fig. 3. Maximum likelihood trees based on the nuclear DNA for (A) *Cerithidea* and (B) *Cerithium*. Tree was constructed using 1023 bp of the 28S gene. Numbers near nodes are the support values for the clade from the different analyses in order: ML/BI (values < 50% not shown).

molecular evolution, the null hypothesis of a molecular clock was not rejected in our combined dataset, suggesting that different rates of molecular evolution do not explain the variation in the evolutionary distances we observed.

Recent studies suggest that not all geminate species pairs were simultaneously separated (see review by Lessios, 2008). Knowlton and Weigt (1998) suggest that ecological differences such as habitat depth may influence the timing of separation. Consistent with this, in our study, the subtidal species *Cerithium uncinatum–C. atratum* exhibited a 30% greater evolutionary distance than the intertidal species *Cerithium stercusmuscarum–C. lutosum* and about twice the distance compared to the high intertidal, mangrove dwelling *C. californica–C. pliculosa* (Fig. 5). *Cerithidea montagnei* and *C. scalariformis* also occur in the high intertidal zone within mangroves. However, the evolutionary distance between these species was about 2.5 times greater than that between *C. californica* and *C. pliculosa* (Fig. 5), suggesting that they split well before *C. californica* and *C. pliculosa*. Interestingly, the distribution of *C. scalariformis* is restricted to the northern parts of the Caribbean (Bequaert, 1942), and thus, *C. scalariformis* may have been confined to this region before the completion of the Isthmus.

It is reasonable to assume that the smallest divergences reflect the final stage of the isthmian formation (Knowlton and Weigt, 1998). However, for molluscs, even the least divergent pairs may have split before the closure of the Isthmus (Lessios, 2008). For example, Marko (2002) calibrated a molecular clock using robust fossil records of ark shells, and concluded that the inferred divergence time for the least divergent pair of geminate ark is 9.9 MYA, which is about 6.8–7.1 million years before the final closure of the Isthmus. Assuming that the substitution rates in closely related groups are not greatly different, comparison of genetic distances between geminate species pairs can help determine which divergence events occurred simultaneously and are thus likely to be contemporaneous with the emergence of the Isthmus. Lessios (2008) reviewed molecular studies for 115 pairs of geminate clades and identified 34 geminate pairs (including 4 gastropod pairs) likely to have been separated at the final stages of completion of the Isthmus. The average Kimura's two parameter (K2P) distance between these geminate gastropods ranges from 7.4% to 9.2% in the COI gene and 0.6% to 3.3% in the 16S gene, and the average divergence in silent sites (K_s) ranges from 23.5% to 30.3% in the COI gene (Lessios, 2008). The average K2P distance between the least divergent geminate pair in our study (*C. californica* and *C. pliculosa*) is 8.7% in COI gene and 1.2% in 16S gene, and the average K_s distance is 30.1% in COI gene. These estimated distances are consistent with other geminate gastropods, suggesting that the split of *C. californica* and *C. pliculosa* corresponded with the final closure of the Isthmus. Thus, we calibrated divergence times with the assumption that *C. californica* and *C. pliculosa* diverged corresponding to the time of the closure of the Isthmus (3.1–2.8 MYA, see Lessios, 2008). Although the estimated divergence time and associated 95% credible intervals of *C. uncinatum–C. atratum* and *Cerithidea montagnei–C. scalariformis* predated the final closure of the Isthmus, those of *C. stercusmuscarum* and *C. lutosum* overlapped with the estimated final closure (Table 3). Thus, we can not exclude the possibility that the split of *C. stercusmuscarum* and *C. lutosum* may also correspond with the final closure of the Isthmus.

Additionally, ancestral polymorphisms can explain differences in evolutionary distances between geminate species pairs (Hickerson et al., 2006; Hurt et al., 2009). Gene divergence can predate

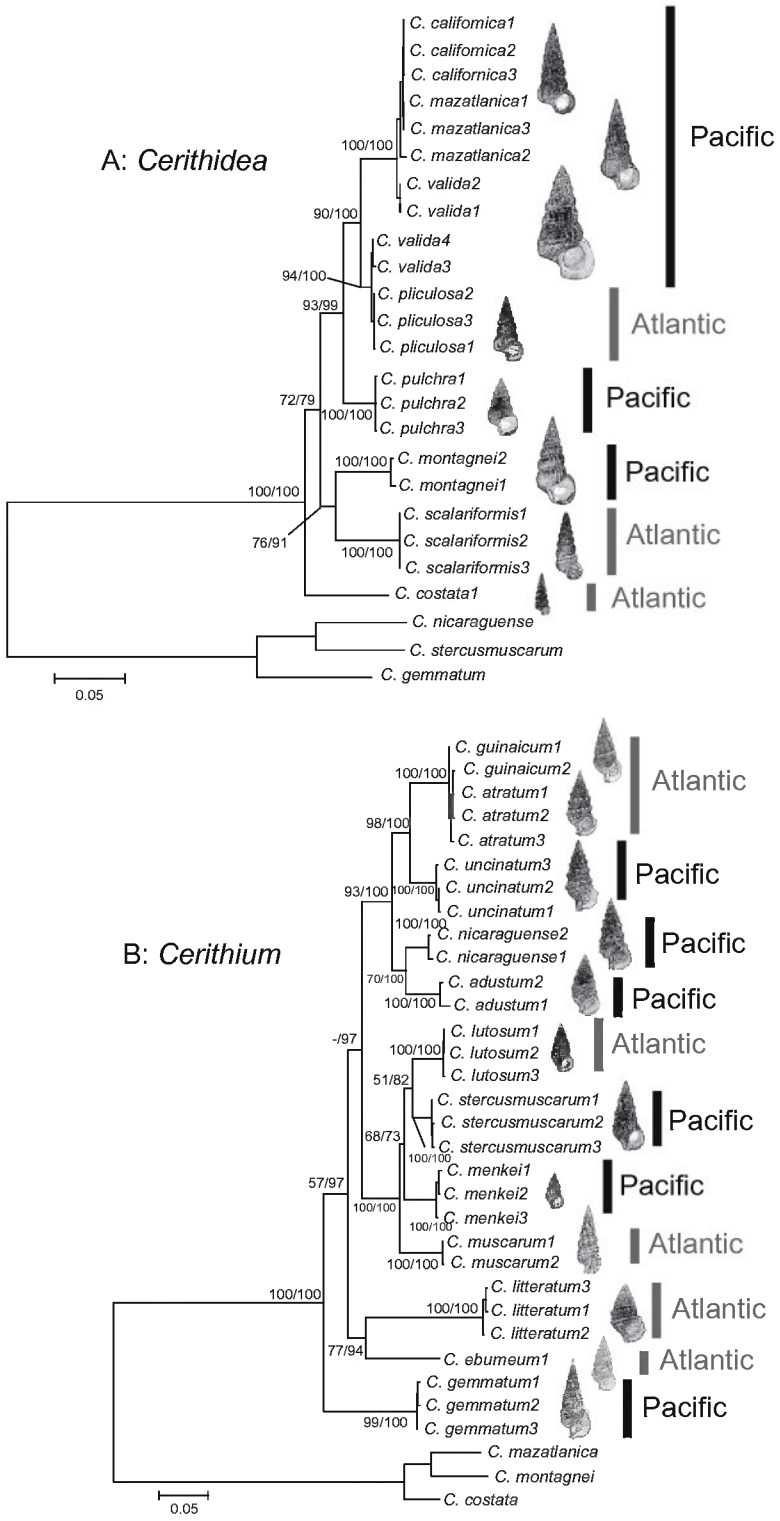

Fig. 4. Maximum likelihood tree based on the combined dataset (2398 bp) for (A) *Cerithidea* and (B) *Cerithium*. Numbers near nodes are the support values for the clade from the different analyses in order: ML/BI (values < 50% not shown).

population divergence due to random sampling of ancestral polymorphisms by descendant populations. For example, Hurt et al. (2009) analyzed one mitochondrial and multiple independent nuclear markers and showed that ancestral polymorphisms can account for much of the variation in mtDNA evolutionary distance between geminate species of snapping shrimp. While this could also explain some of the variation in evolutionary distances in our study, we lack the required detailed information of population size and multiple independent nuclear markers to fully evaluate this hypothesis at the moment.

4.5. Hybridization of geminate species

Genetic divergence is a precursor for allopatric diversification, but reproductive isolation ultimately determines speciation. Reproductive isolation may be reinforced between closely related

Table 3
The divergence times between geminate species pairs estimated using BEAST.

Geminate species pairs		Divergence time	95% HPD
Pacific	Atlantic	(MYA)	(MYA)
C. californica	*C. pliculosa*	3.1–2.8[a]	–
C. stercusmuscarum	*C. lutosum*	3.7	5.0–2.6
C. atratum	*C. uncinatum*	5.1	6.7–3.5
C. montagnei	*C. scalariformis*	6.4	8.3–4.5

[a] Calibration point: closure of the Isthmus of Panama.

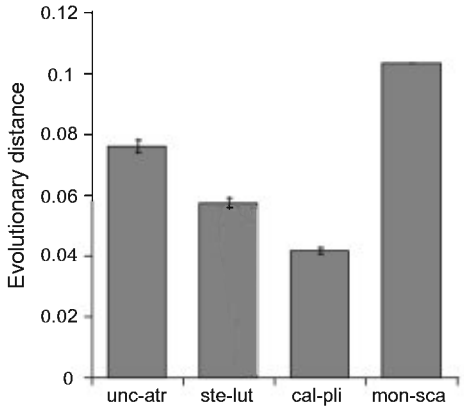

Fig. 5. Average evolutionary distances of the potential geminate species pairs in the combined dataset. The distances were estimated under the best evolutionary model (see Table 2). Individuals suspected of hybridization (*C. valida* 3 and 4) were removed from the analyses. The geminate species pairs, *Cerithium uncinatum* and *C. atratum* are indicated by unc-atr, *C. stercusmuscrum* and *C. lutosum* are indicated by ste-lut, *Cerithidea californica* species complex and *C. pliculosa* are indicated by cal-pli, and *C. montagnei* and *C. scalariformis* are represented by mon-sca. Error bars represent ±1 SE.

sympatric species, whereas allopatric geminate species pairs may not have developed complete reproductive isolation because reproductive characters may diverge faster in sympatric species pairs than geminate pairs (Lessios, 2008). This has been demonstrated in gobioid fishes in the genus *Bathygobius* (Rubinoff and Rubinoff, 1971) and sea urchins in the genus *Echinometra* in Central America (Lessios and Cunningham, 1990; McCartney and Lessios, 2002). Similarly, our data suggest that Atlantic *C. pliculosa* could have dispersed to the Pacific after the closure of the Isthmus and hybridized with Pacific *C. californica* since some Pacific snails have similar mitochondrial haplotypes as the Atlantic snails (Figs. 1A and 2A). Further, it is noteworthy that an individual Pacific *C. californica* had a similar mitochondrial haplotype to Atlantic *C. pliculosa* (Figs. 1A and 2A) and also had an identical 28S genotype to sympatric Pacific *C. pulchra* (Fig. 3A). These inconsistencies in mitochondrial and nuclear gene phylogenies suggest that three related species, *C. californica*, *C. pliculosa* and *C. pulchra* might produce hybrids. However, we found no evidence of hybridization between "pure" *C. californica* (i.e., not hybridized with *C. pliculosa*) and sympatric *C. pulchra*. Thus, recent dispersal and hybridization of *C. pliculosa* with *C. californica* might facilitate sympatric hybridization that would not occur otherwise. Given the increasing rate of human-mediated biological invasions it is necessary to consider the potential dissolution of reproductive barriers between current and future sympatric species. These mud snails provide an ideal opportunity to investigate this possibility.

4.6. Concluding remarks

Shell morphology is often plastic and can vary across different environments. Thus it is important to verify geminate species pairs through genetic comparisons. Three putative geminate species pairs were not closely related in our phylogeny and even though they are morphologically similar they are not sister species. Additionally, while near-shore taxa are better candidates for molecular clock calibrations, sequence divergences among near-shore geminate pairs of snail species can vary more than two-fold, suggesting caution when selecting species pairs to estimate rates of evolution. Finally, consistent with other studies across the Isthmus of Panama (Rubinoff and Rubinoff, 1971; Lessios and Cunningham, 1990; McCartney and Lessios, 2002), our findings suggest that reproductive isolation between geminate species is not always complete and that recent dispersal and subsequent hybridization may provide a mechanism to break reproductive barriers between sympatric species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.012.

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