

Integrating fossils and molecules to study cupuladriid evolution in an emerging Isthmus

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Abstract Fossils and genes represent two principal sources of data for studying evolutionary biology, but they are rarely unified. The aim of this paper is to integrate the fossil and molecular records of Tropical American cupuladriid bryozoans to elucidate their evolutionary history. Molecules and fossils broadly concur in determining the timing of species divergences, and point to seaway constriction associated with the emergence of the Isthmus of Panama as a driver of speciation. We discover that although cross-Isthmian isolation of taxa was important, strong isolating mechanisms also existed within the southwestern Caribbean (SWC), caused by increasing physical and environmental heterogeneity as inter-oceanic straits constricted in the Late Pliocene. During this time of rapid environmental change and instability, recently diverged species pairs existed in locally separated habitats for around 2 million years, only to co-exist after final closure of the seaway. This pattern is consistent with a model of allopatric speciation caused by local isolating mechanisms followed by secondary contact. Fossils also reveal the trajectories of reproductive life history and morphology during and after species divergences. In the SWC, all extant species started to shift from clonal to aclonal reproduction immediately in response to changing oceanographic conditions. However, it took another million years for colonies to gain skeletal strength, a trait that reduces cloning by fragmentation, suggesting that the appearance of advantageous traits was delayed by 1–2 million years and only arose after the process of allopatric speciation had run its course. Changes in colony shape, height and size also appear to lag 2 million years, but may reflect exploitation of the diverse sedimentary environments created as reefs proliferated at this time.

Keywords Cupuladria · Isthmus of Panama · Morphological divergence · Fossil · Molecules · Evolutionary ecology

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Introduction

The Central American Seaway (CAS) formed over 150 million years ago (Ma; Smith and Tipper 1986), establishing a more or less continuous tropical marine connection between the Pacific and Atlantic oceans. Then, only 15 Ma, the tectonic processes reversed and the seaway began to narrow. As it did, the submarine arc of southern Central America collided with South America whilst sub-aerial terrains were formed by enhanced volcanic activity (Duquecaro 1990; Coates and Obando 1996), both resulting in the slow but steady constriction of the Central American Seaway (CAS), the formation of narrow and shallow inter-oceanic straits and the eventual complete isolation of the Tropical Eastern Pacific (TEP) from the southwestern Caribbean (SWC) at around 3 Ma.

The resulting land mass we know today as the Isthmus of Panama caused profound changes in the seascapes of Tropical America, altering ocean currents, triggering major ecological shifts, and stimulating speciation and heightening extinction events across a wide array of marine organisms (Haug and Tiedemann 1998; Jackson and D'croz 1999; Todd et al. 2002; Bartoli et al. 2005; O'Dea et al. 2007; Smith and Jackson 2009). The separation of once-continuous populations of marine organisms, the differing marine conditions across the Isthmus today (Lessios 1981) and the richly fossiliferous marine sediments preserved on both coasts of the Isthmus that are set within a stratigraphically well-constrained system (Collins and Coates 1999) make the Isthmus of Panama a unique place to study evolutionary ecology (Jackson and D'croz 1999; Lessios 2008).

In this study we unite fossil and molecular data from cupuladriid bryozoans to understand their evolution through the last 10 million years to explore (1) species divergences in a spatial and temporal framework and (2) the trajectories of morphological traits in closely related species during and following divergence.

Tectonics, transitions and trials in Tropical America

The emergence of the Isthmus of Panama and closure of the inter-oceanic straits ~ 3 Ma was a slow process of complex geological and tectonic changes (Duquecaro 1990; Coates and Obando 1996). Lower Central America in the Late Miocene existed as an intricate archipelago with islands and several straits connecting the TEP with the SWC (Coates and Obando 1996; Collins et al. 1996b). Three principal straits maintained trans-oceanic connection: the Tempisque-San Carlos-North Limon Corridor in Nicaragua and Costa Rica, the Canal Corridor in central Panama, and the Atrato Corridor in the Darién of Panama and Colombia (Coates et al. 1992, 2003, 2004, 2005; Coates and Obando 1996; Collins et al. 1995, 1996a, b). These three straits dried up from west to east through time, with the Atrato corridor representing the ultimate TEP-SWC strait. Rapid shallowing occurred in the Late Miocene, with the Canal (Duquecaro 1990; Collins et al. 1996b) and Tempisque-San Carlos-North Limon (Coates et al. 2003) Corridors almost closing, while the Atrato Corridor remained fairly deep at around 200–500 m (Coates et al. 2004). A substantial geographic barrier was therefore in place during the Late Miocene, as evidenced by diverging coral and benthic foraminifera assemblages (Duquecaro 1990; Collins et al. 1996b) and molecular data from a variety of taxa (Knowlton and Weigt 1998; Marko 2000), particularly deep-water taxa.

However, this initial restriction of inter-oceanic waters was temporary; cross-isthmian straits deepened substantially around 7–6 Ma as depositional depths in the canal basin deepened from ~ 35 to ~ 200 m (Coates et al. 2003), and similar deepening events occurred in the coeval Chucunaque and Uscari formations in Darien and Costa Rica,

respectively (Coates et al. 2003, 2004). From the Late Miocene and into the Early Pliocene, oceanic connectivity began to be impeded once more. Nutrient levels began to fall in the SWC as Pacific waters stopped entering the Caribbean (O'Dea et al. 2007). Consequently, environmental and habitat heterogeneity and carbonate deposition increased rapidly as reefs began to proliferate (Todd et al. 2002; Johnson et al. 2007, 2008; O'Dea et al. 2007; Smith and Jackson 2009).

Increasing habitat complexity and fragmenting shallow marine ecosystems have long been considered important stimuli to diversification (see Renema et al. 2008), and such processes may have provided opportunity and time for species isolation and hence allopatric speciation in the SWC (Jackson et al. 1996), although no studies have specifically addressed this subject. Indeed, taxonomic diversity in most groups increased in the Late Miocene and Early to Late Pliocene as origination rates of taxa peaked (Jackson et al. 1993; Cheetham and Jackson 1996; Collins 1996; Knowlton and Weigt 1998; Budd and Johnson 1999; Marko 2000; Smith and Jackson 2009). After final closure of the CAS, origination rates fell dramatically to near zero in most groups, and a regional mass extinction perturbed the entire Caribbean benthos between 3 and 1 Ma (Vermeij and Petuch 1986; Allmon 1992, 2001; Budd and Johnson 1999; Jackson and Johnson 2000, 2001; O'Dea et al. 2007; Smith and Jackson 2009).

Cupuladriid bryozoans

Diversity and abundance

Cupuladriids are a moderately diverse family of tropical and sub-tropical cheilostome bryozoans and are extremely abundant in Tropical American shelf seas (O'Dea et al. 2004). They are represented by an exceptionally rich Neogene fossil record demonstrating their lasting importance as members of the epibenthic fauna on instable sands and muds in Tropical American waters (Cook and Chimonides 1983; McKinney and Jackson 1989; Cheetham and Jackson 1996; Cheetham et al. 1999; O'Dea et al. 2008; O'Dea and Jackson 2009). Of the three known genera of free-living Cupuladriidae (*Discoporella*, *Cupuladria* and *Ruiserella*), *Discoporella* and *Cupuladria* are diverse and abundant in Panama in both the present and fossil record (Cheetham et al. 1999; O'Dea et al. 2004; O'Dea and Jackson 2009). Present-day species diversity is higher in the SWC (12 extant species) than the TEP (4 extant species; Dick et al. 2003; Herrera-Cubilla et al. 2006, 2008).

Mode of life

Unlike most bryozoans, cupuladriids live freely, unattached to a substrate (Fig. 1). Their dome-shaped colonies possess elongate setae, growing from specialized zooids, that are used for defense and mobility: to either unbury themselves or move over the soft sands they inhabit (Cook 1963; Winston 1988; Cook and Chimonides 1994; O'Dea 2009; Fig. 1e).

Morphology controls mode of reproduction

Like most colonial organisms (Lasker 1984) cupuladriids are capable of propagating new colonies both sexually (from larvae) and clonally (via fragmentation and regeneration; Winston 1988; Håkansson and Thomsen 2001). The frequency of each mode of

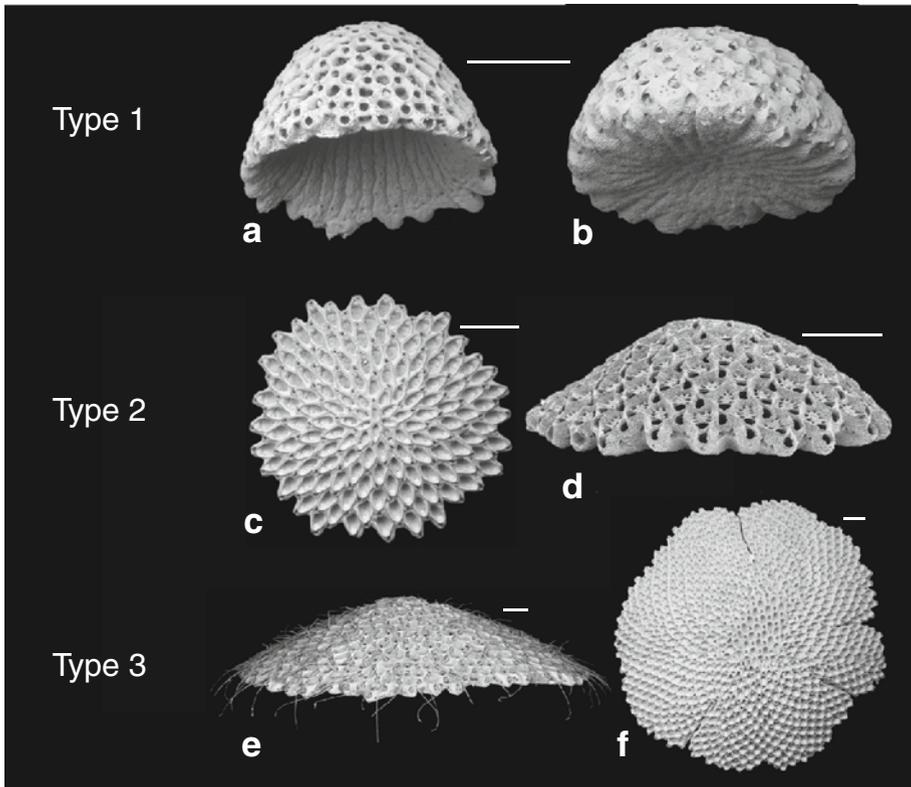


Fig. 1 Variety of colony forms in cupuladriid bryozoan species. **a–b** *Type 1*; typically *short*, conical and heavily calcified; rarely fragments; reproduces mostly sexually. **c–d**, *Type 2*; moderately high in shape, size and calcification of colonies; fragments casually; reproduces clonally and sexually. **d–e**, *Type 3*; flat, large, lightly calcified colonies; fragments effortlessly or possesses special morphologies to enhance fragmentation or budding; reproduces mostly clonally. *Scale bar* = 1 mm. SEM images courtesy of P.D. Taylor

reproduction varies considerably across species and is highly correlated with colony form and construction (O’Dea et al. 2004, 2008). Most species have simple colonies that fragment by happenstance, whilst others have morphologies that either strongly promote or strongly curb clonal reproduction (e.g. Fig. 1). Species that readily clone tend to have large, flat, lightly calcified colonies that fragment easily through disturbances on the sea floor, suggesting that species control rates of cloning by altering the thickness of their colonies. Indeed, the level of calcification is significantly correlated with rate of cloning amongst species (O’Dea et al. 2004). Some species are able to increase rates of cloning considerably more than would happen through accidental fragmentation, by means of colonial budding and autofragmentation, which result from distinct morphologies and growth modes (Fig. 1f; Marcus and Marcus 1962; Håkansson and Thomsen 2001; O’Dea 2006; O’Dea et al. 2008). Some species take the opposite approach and prevent cloning by producing small, squat, heavily calcified colonies that rarely fragment (O’Dea et al. 2004, 2008; Fig. 1a, b). Each of these modes is preserved in the calcified skeleton and hence permits the exploration of the evolution of reproductive life histories

using the exceptional cupuladriid fossil record (O’Dea et al. 2008; O’Dea and Jackson 2009).

Sex is best when food is scarce

Areas with high planktonic productivity have a greater proportion of species that reproduce clonally (O’Dea et al. 2004). Across the Isthmus of Panama today, most TEP species predominantly use clonal reproduction, whilst SWC species tend to reproduce sexually consistent with the greater planktonic productivity of the TEP. Through time, fossils from the SWC reveal a species-level turnover from clonal to sexual reproduction as upwelling declined and food levels fell. What’s more, several predominantly clonal species survived this environmental change by altering their life histories and shifting to more sexual reproduction. Those that maintained high levels of cloning were less suited to the new conditions, dropped in abundance and distribution and eventually went extinct (O’Dea and Jackson 2009). Species that span environments with differing food levels clone more in areas with high food (O’Dea et al. 2004), and some species clone specifically to take advantage of seasonal blooms in phytoplankton (O’Dea 2006). High rates of cloning appear to be viable only when food levels are sufficient to support both the added vegetative growth and quick regeneration following fragmentation that are required for cloning (O’Dea 2006).

Rationale

O’Dea and Jackson (2009) revealed that the origination and extinction of cupuladriid species in the SWC were strongly determined by reproductive life history. As food levels fell, newly originating species were all predominantly asexual, surviving species evaded extinction by reducing rates of cloning, and species that maintained high levels of cloning were doomed to extinction (O’Dea and Jackson 2009). Increased calcification levels were implicated in the shift to greater sexual reproduction within species, mirroring the strong negative correlation between colony strength and rate of cloning observed amongst modern species (O’Dea et al. 2004). In the same way, O’Dea et al. (2004) concluded that the small, squat colonies produced by some species were a trait designed to reduce fragmentation, whilst large flat colonies enhanced fragmentation and hence clonal reproduction. However, O’Dea (2009) showed that colony form in modern assemblages strongly relates to niche occupation and survival under different sedimentary environments.

Several questions remain unanswered. (1) When and under what conditions did new species arise and how were they related to surviving SWC/TEP species? (2) How constrained were life histories in the phylogenetic and environmental contexts? (3) Did the evolution of the skeletal variation seen today arise during species divergences, oceanographic change and increasing habitat heterogeneity?

We approach these issues with a combined molecular and fossil analysis, an approach that is infrequently applied yet has great potential for revealing evolutionary patterns and processes (Fukami et al. 2004; Williams 2007; Renema et al. 2008; Quental and Marshall 2010). By establishing phylogenetic relationships among all extant cupuladriids from both sides of the Isthmus of Panama using molecular data, we selected pairs of closely related species with excellent fossil records and examined the regional biogeography and sequence of changes in reproductive mode and colony form in these pairs during and after their divergence.

Materials and methods

Specimen collection

50,378 fossil and modern colonies of the following species were collected from 1317 Neogene bulk collections and 171 dredge samples from Pacific and Caribbean shelf waters: *Cupuladria biporosa* (13,904), *C. surinamensis* (9,837), *C. exfragminis* (1,106), *C. incognita* (7,354), *C. cheethami* (106), *Dicoporella cookae* (6,965), *D. scutella* (3,552) and *D. peltifera* (7,554). Sampling in the SWC between 5 Ma and the present was excellent, with high spatio-temporal replication and age ranges of sediments typically resolved to \pm a few hundred thousand years, whereas samples between 10 and 6 Ma were much fewer and less well resolved, with age-range estimates typically \pm 1 million years (Cheetham et al. 1999; O’Dea et al. 2007). See O’Dea and Jackson (2009) for details of the fossil locations (faunules) used in the SWC. Fossil TEP cupuladriids were collected by bulk sampling from three formations: Punta la Peña (E. Pliocene), Burica (Pliocene) and Armuelles (Late Pliocene–Pleistocene; Collins et al. 1995). For molecular analyses, live cupuladriids were collected from both coasts of the Isthmus of Panama by dredge and were stored in alcohol.

Morphological analyses

All well-preserved colonies were classified to species using morphometric variables that were established through multivariate approaches to discriminant analysis (Herrera-Cubilla et al. 2006, 2008) and supported by molecular discrimination (Dick et al. 2003 and this study).

Using the Neogene fossil record of the SWC and TEP (Cheetham et al. 1999; O’Dea and Jackson 2009), we traced morphological and life history traits from species origins to present day for five species pair determined from the molecular data. Pairs were chosen for which we had established evolutionary relationships and divergence times (Table 1; Fig. 2) and for which a good fossil record exists.

Each colony was measured (height, and maximum and minimum diameters, from which the median diameter was calculated) and identified as either clonal or sexual (aclonal) following the criteria of O’Dea and Jackson (2009). Colony shape (height divided by median colony diameter) was calculated for each colony. Percent clonal colonies, mean colony height and mean colony shape were calculated for each species per time bin (10–5, 5–4, 4–3, 3–2, 2–1, 1–0 Ma). The calcification index for each species per time bin was calculated by summing the weight of all colonies within each time bin and dividing that by the total surface area of all colonies within that bin, following the methods described in O’Dea et al. (2004) and O’Dea and Jackson (2009). Colony shape, size, reproductive mode and calcification index are morphological traits with ecological significance (see O’Dea 2009) and are different from morphological characters (typically zooidal traits) used for taxonomic purposes (see Herrera-Cubilla et al. 2006, 2008).

Species categories

Major shifts in life history across species divergences were explored by grouping extant species on the basis of shape, size, reproductive mode and calcification. Cupuladriid species can be broadly categorized into three types (Table 2; Fig. 1), as follows. *Type 1*

tends to have heavily calcified, conical, compact colonies that predominantly reproduce sexually (aclonally). *Type 2* tends to have moderately calcified, intermediate-sized colonies that reproduce clonally and sexually at roughly equal frequencies. *Type 3* tends to have thinly calcified, slightly flattened, large colonies that predominantly reproduce sexually (Fig. 1).

Molecular analyses

Five setae were dissected from the periphery of each colony for DNA extraction. This method considerably reduced the amplification of epibionts that would otherwise occur if partial or entire colonies were used (see Dick et al. 2003). For phylogenetic analyses, we amplified a fragment of the mitochondrial 16S gene as well as a fragment of the nuclear 18S gene. DNA extractions, primers and PCR conditions to amplify the 16S fragment are described in Dick et al. (2003). The 18S fragment was amplified initially using primers based on *Bugula turrita* 18S sequence (18sFor 5'-CCA CCA GGA GTG GAG CCT-3', 18sRev 5'-TCA ATC GGT AGT AGC GAC-3', NCBI Accession number AY21044; Passamaneck and Halanych 2006). Once valid sequences were obtained from several cupuladriid species, we designed cupuladriid specific primers Cup18sFor (5'-CCA CCA GGA GWR GAG CCT-3') and Cup18sRev (5'-YTC AAT CGG TAG TAG CGA CG-3') to amplify the 18S fragment from all cupuladriids used in this study. PCR conditions to amplify the 18S fragment were as follows: 35 cycles of 95°C for 1 min, 95°C for 45 s, 52°C for 30 s and 72°C for 1.30 min, with a final extension at 72°C for 5 min. Following cleaning, products were sequenced in an ABI3130 XL automated sequencer (ABI). All sequences were checked for errors and edited by using Sequencher 3.1 (Gene Codes Corporation). Consensus sequences, representing 3 individuals for each species except for *C. cheethami*, *C. sp. 10* and *D. peltifera* (only one colony was available), were used for molecular analyses (only a 280 bp fragment of *D. peltifera* 16S was used).

Phylogenetic analyses

Our analysis is an extension of the detailed 16S phylogeny produced by Dick et al. (2003), at which time cupuladriids species used in their study had not yet been described. We added an additional five species (*C. incognita*, *C. cheethami*, *C. sp. 10*, *C. sp. 14*¹ and *D. sp. P1*, see O'Dea and Jackson 2009; O'Dea 2009) and used partial sequences of the 16S and 18S gene as a combined dataset of 961 nucleotides for phylogenetic reconstruction. All sequences were manually aligned using MacClade v4.06 (Maddison and Maddison 2000). Gaps in the 16S gene were treated as 'missing data' that are optimally reconstructed by the program during phylogenetic reconstruction. *Bugula nerita* was used as an outgroup taxon. While the 16S fragment provided sufficient resolution to determine species relationships (see Dick et al. 2003), we retained the 18S fragment because it resolved and improved support values for deeper divergences. The appropriate evolutionary models for phylogenetic analyses using our dataset were selected using Mr. ModelTest (Nylander 2004), a program that estimates the best-fit model of nucleotide substitutions for a given dataset. For our analysis GTR + I + G for 16S and GTR + G for 18S were determined to be the best-fit models. Phylogenetic reconstructions were then done using Mr. Bayes v3.1, a program for Bayesian inference of phylogeny that uses a combination of Markov chain Monte Carlo (MCMC) and Metropolis-coupled Markov chain Monte Carlo sampling methods (Ronquist

¹ *C. sp. 14* is synonymous with *C. sp. nov. 'gigante'* in O'Dea et al. (2008) and O'Dea (2009).

and Huelsenbeck 2001). Analyses in Mr. Bayes were run for 6 million generations, with a sampling frequency of 100. The first 25% percent of trees were discarded; each run retained 30,001 trees. Mr. Bayes automatically analyzes the data in two independent runs, therefore a total of 60,002 trees was used to estimate phylogenetic relationships and to approximate posterior probabilities of each clade. Convergence between the two independent runs in Mr. Bayes (reflecting increasing similarity of trees from independent runs) was determined by examining the standard deviation of split frequencies (<0.001) and potential scale-reduction factors (1.0). To estimate divergence times we used BEAST 1.5.2, a software package that implements Bayesian MCMC algorithm (Drummond and Rambaut 2007). An important feature of BEAST is the estimation of calibrated phylogenies based on relevant paleontological knowledge. Appropriate calibrations are necessary to convert genetic divergence measures into units of geological time and fossil data have played an important role in calibrating evolutionary rates and divergence times (Benton and Donoghue 2007) but see Miura et al. (2010) who used similar methods without fossil calibrations for phylogenetic investigations of intertidal snails). We used a GTR + I substitution model for the combined 16S and 18S dataset for analysis in BEAST. Tracer 1.4.1, a program for analyzing output files generated by Bayesian MCMC runs were used to determine the optimal parameters for our analyses in BEAST, particularly the number of generations and tree priors. Tracer indicated that our dataset was not clock-like (uclid. SD and coefficient of variation were close to 1.0 rather than 0, indicating rate heterogeneity between branches). Accordingly, we ran our analyses for 80 million generations under an uncorrelated lognormal relaxed molecular model that allows evolutionary rates to vary across branches of the tree. Tree priors (oldest fossil occurrence of species, as per O'Dea and Jackson 2009) used to calibrate evolutionary rates are provided in Table 1. Resulting phylogenetic trees were viewed and edited with FigTree 1.2.3 (Drummond and Rambaut 2007), a program that allows graphical viewing and image editing of phylogenetic trees. It must be noted that no morphological characters were used in phylogenetic reconstructions in this study, however, the molecular phylogeny produced by Dick et al. (2003) as well as ours supports species classifications based on morphological characters from fossil and recent species (Herrera-Cubilla in prep).

Co-occurrences of species pairs

To explore patterns of sympatry and allopatry in species pairs during and subsequent to their purported divergences (as estimated from fossils and molecules), we calculated co-occurrence as the percentage of samples in which both species in a pair occurred relative to all samples in which they could potentially co-occur given their age range. We calculated this in two time bins; (1) 4.5–3.0 Ma, corresponding to the time of greatest environmental change during final shoaling and closure of the Isthmus of Panama (O'Dea and Jackson 2009), and (2) 3.0–2.0 Ma, which is post-closure and represents a time of established environmental stability. We did not include the Recent in the post-closure time bin because dredge samples potentially contain several environmental types. Fossil bulk samples are well constrained spatially and therefore environmentally because each faunule represents a package of lithologically-similar sediment amounting to a few meters of section that can be treated as an ecological unit in space and time (see O'Dea et al. 2007) but are potentially time averaged. The effects of time averaging on reconstructing past communities are not considered excessive (Kidwell 2001, 2005; Jackson and Erwin 2006) and in this case were not expected to change over time, as the types of samples taken and general depositional environments were no different through time in our study. Thus, any

changes through time in the percentage co-occurrence of species pairs are likely to reflect changes in relative true co-occurrences in species pairs, and can therefore be used to explore relative levels of sympatry and allopatry. We did not calculate co-occurrences for trans-isthmian species pairs given that they became separated by the Isthmus and went extinct in the Caribbean (O’Dea and Jackson 2009).

Results

Phylogenetic relationships and estimates of divergence times

Phylogenetic reconstruction by Bayesian inference (Fig. 2) reveals the phylogenetic relationships of fourteen genetically distinct cupuladriids species. In *Cupuladria*, the *C. surinamensis* group is comprised of *C. surinamensis* and a recently derived species *C. sp. 14* both of which are limited to the Caribbean coast. The *C. biporosa* group is the most speciose, comprising five species (*C. biporosa*, *C. cheethami*, *C. incognita*, *C. exfragminis* and *C. sp. 10*), among which *C. exfragminis* and *C. incognita* are trans-isthmian sister species. The only well-resolved monophyletic group in *Discoporella* is that of *D. cookae*, which contains two pairs of species separated by the Isthmus: *D. scutella* and *D. peltifera* in the Caribbean, and *D. cookae* and the recently derived *D. marcusorum* in the Pacific. *D. bocasdeltoroensis*, *D. terminata* and *D. sp. P1* lie in an unresolved polytomy (also see Dick et al. 2003).

There is good agreement between the fossil and molecular estimates of divergence times (Table 1; Fig. 2). Both records indicate that most cupuladriid species diverged before final closure of the Isthmus (Fig. 2; also see Dick et al. 2003). The split between *C. biporosa* and *C. surinamensis* is the oldest, dating from the mid-Miocene ~15 Ma, coinciding with the original radiation of cupuladriids into the Americas (Cook and Chimonides 1983). *C. biporosa* has been reported to occur in Middle Miocene sediments in the Dominican Republic (Alan Cheetham; NMITA). *D. bocasdeltoroensis* is also an old species but has no known fossil record outside Late Miocene sediments in Panama. Two

Table 1 Estimates of divergence times of cupuladriid species pairs

Species pairs	Divergence time (Ma)	95% HPD (Ma)	Oldest fossil occurrence calibrations (Ma)
<i>D. cookae</i> versus <i>D. scutella</i> / <i>D. peltifera</i>	7.56	9.8–5.4	<i>D. cookae</i> (8.2)
<i>D. scutella</i> versus <i>D. peltifera</i>	4.36	5.2–3.4	<i>D. scutella</i> and <i>D. peltifera</i> (4.23)
<i>D. cookae</i> versus <i>D. marcusorum</i>	2.37	2.7–1.8	<i>D. marcusorum</i> (2.1)
<i>C. biporosa</i> versus <i>C. surinamensis</i>	14.61	16.5–12.8	<i>C. surinamensis</i> (12)
<i>C. biporosa</i> versus <i>C. incognita</i> / <i>C. exfragminis</i>	6.8	8.6–4.9	<i>C. biporosa</i> (10), <i>C. incognita</i> (4.23)
<i>C. biporosa</i> versus <i>C. cheethami</i>	4.38	5.3–3.3	<i>C. cheethami</i> (4.23)
<i>C. incognita</i> versus <i>C. exfragminis</i>	4.45	5.3–3.6	<i>C. incognita</i> (4.23)
<i>C. surinamensis</i> versus <i>C. sp. 14</i>	1.61	3.0–0.2	No fossil record of <i>C. sp. 14</i>
<i>C. biporosa</i> versus <i>C. sp. 10</i>	1.65	3.0–0.3	No fossil record of <i>C. sp. 10</i>

Fossil calibrations are from the SWC, except for *D. marcusorum* whose oldest fossil occurrence is TEP HPD highest posterior density

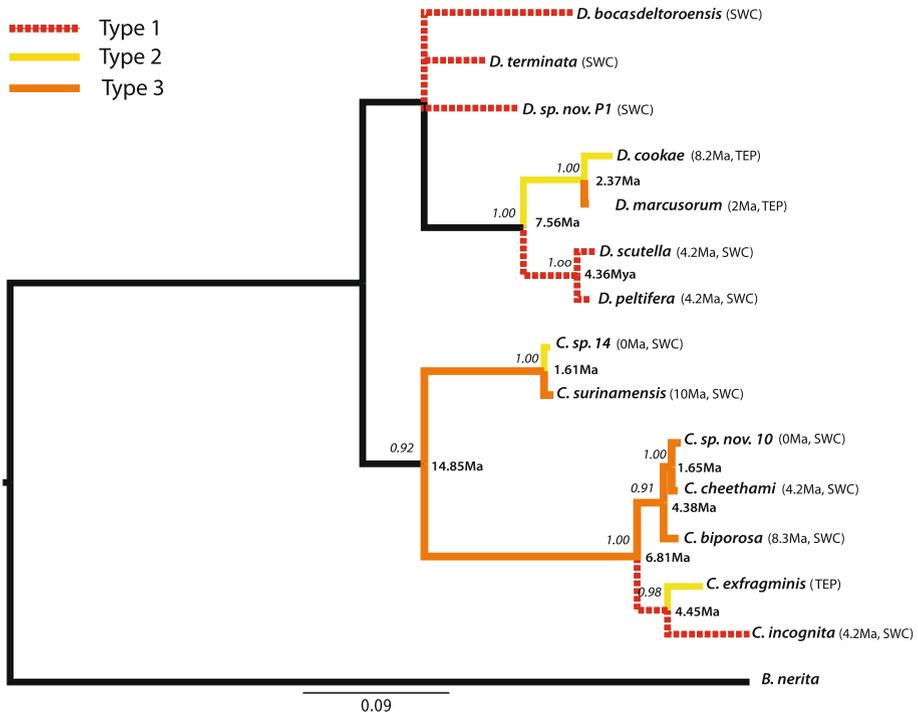


Fig. 2 Gross life history and colony form mapped onto a molecular phylogenetic tree of extant cupuladriid bryozoans on either side of the Isthmus of Panama. *Numbers* at nodes are median divergence times estimated by molecular analyses (Table 1). *Italicized numbers* near nodes—posterior probabilities (support values below 60 are not shown). *Numbers in parentheses*—earliest fossil occurrence. SWC Southwestern Caribbean, TEP Tropical Eastern Pacific

contemporaneous radiations occurred in the Late Miocene: one at ~ 7.6 Ma in *Discoporella* gave rise to the *D. cookae* clade, and the other at ~ 6.8 Ma in *Cupuladria* produced the *C. biporosa* clade. The fossil record for *D. cookae* in Panama dates back to ~ 8 Ma, but *D. cookae* went extinct in the Caribbean at ~ 3 Ma whereas Pacific populations survived (O’Dea and Jackson 2009), and *D. marcusorum* diverged from *D. cookae* ~ 2.3 Ma. The divergence between *D. scutella* and *D. peltifera* occurred ~ 4.4 Ma, and both appeared in the fossil record ~ 4.3 Ma (O’Dea and Jackson 2009). Whether Caribbean populations of *D. cookae* diversified and survived as *D. scutella* and *D. peltifera* requires further detailed study, but the existence of an extinct sister lineage to *D. cookae* from which *D. scutella* and *D. peltifera* diversified cannot be ruled out. The same scenario applies to *C. biporosa* clade. Since its divergence from *C. surinamensis* in the mid-Miocene, the *C. biporosa* lineage appears to have split at least four times to produce five extant species, including *C. biporosa* (Fig. 2). Inclusion of extinct species would probably resolve the apparent difference in divergence rates between the *D. cookae* and *C. biporosa* clades, although in the SWC the number of known extinct *Cupuladria* is less than half that of *Discoporella* (O’Dea and Jackson 2009). The Late Miocene radiation in *Cupuladria* ~ 6.8 Ma gave rise to two groups. One contains *C. biporosa* and the sibling species *C. sp. 10* and *C. cheethami*. The *C. biporosa*–*C. cheethami* split is placed at ~ 4.38 Ma and *C. sp. 10* diverged from *C. cheethami* < 2.0 Ma. The other group contains transisthmian species *C. exfragminis* and *C. incognita*, which separated ~ 4.45 Ma. Interestingly, *C. cheethami* also diverged from

Table 2 Summary of gross life history and colony forms in Tropical American cupuladriid bryozoans

Species	Modern distribution	Colony form	Calcification	Dominant mode of reproduction	Type
<i>Discoporella</i>					
<i>D. bocasdeltoroensis</i>	SWC	Compact	High	Aclonal	1
<i>D. terminata</i>	SWC	Compact	High	Aclonal	1
<i>D. sp. P1</i>	TEP	Compact	High	Aclonal	1
<i>D. cookae</i>	TEP	Large	Low	Clonal	3
<i>D. marcusorum</i>	TEP	Medium	Moderate	Clonal/aclonal	2
<i>D. scutella</i>	SWC	Compact	High	Aclonal	1
<i>D. peltifera</i>	SWC	Compact	High	Aclonal	1
<i>Cupuladria</i>					
<i>C. sp. 14</i>	SWC	Large	Low	Clonal	3
<i>C. surinamensis</i>	SWC	Medium	Moderate	Clonal/aclonal	2
<i>C. sp. 10</i>	SWC	Medium	Moderate	Clonal/aclonal	2
<i>C. cheethami</i>	SWC	Medium	Moderate	Clonal/aclonal	2
<i>C. biporosa</i>	SWC	Medium	Moderate	Clonal/aclonal	2
<i>C. exfragminis</i>	TEP	Large	Low	Clonal	3
<i>C. incognita</i>	SWC	Compact	High	Aclonal	1

SWC southwestern Caribbean, TEP Tropical Eastern Pacific

C. biporosa ~4.38 Ma, almost the same time as the *C. exfragminis*-*C. incognita* split, but both *C. biporosa* and *C. cheethami* are Type 2 species, suggesting isolation in similar environments.

Interspecific shifts in life histories and morphologies

Type 1 and Type 2 species tend to dominate the SWC, whereas Type 3 species dominate the TEP (O’Dea 2006; O’Dea et al. 2004, 2008). There are exceptions, such as the TEP species *D. sp. P1*, which is thickly calcified, highly aclonal, and of Type 1, and the SWC species *C. sp. 14*, a Type 3 species, though occurring only as a single, small, geographically restricted population.

Discoporella species belong to Type 1 with the exception of *D. cookae* (Type 3), which went extinct in the SWC, and *D. marcusorum* (Type 2), which is and has always been restricted to the TEP; in contrast, most *Cupuladria* species belong to Type 2 or 3, except for *C. incognita* (Type 1). Radical shifts across life history categories (i.e. a shift across two types) associated with divergences occur only in transisthmian pairs of both genera; i.e. *D. cookae* versus *D. scutella*/*D. peltifera* and *C. exfragminis* versus *C. incognita* (Fig. 2). Other shifts were moderate (i.e. from Type 1–2, or 2–3 and vice versa).

Intraspecific shifts in life histories and morphologies

In general, SWC species reduced the rate of cloning beginning 5–4 Ma, increased calcification 3–2 Ma and became smaller and more squat 2–1 Ma. Conversely, changes in TEP species were relatively less (Fig. 3).

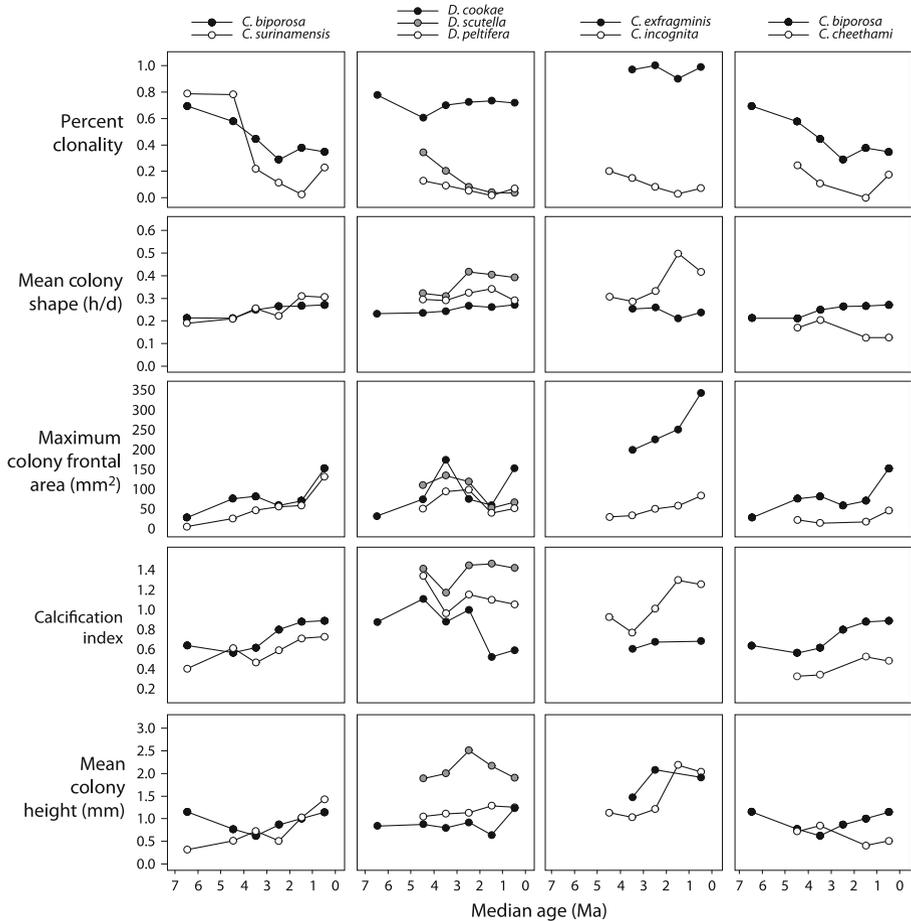


Fig. 3 Temporal patterns of life history (percent clonality) and colony form (colony height, colony shape, calcification and colony size) for eight cupuladriid species over the last 10 million years. Species are grouped with their closest relatives as determined by molecular phylogeny to compare trends in species pairs

Geographic isolation of species pairs in the SWC

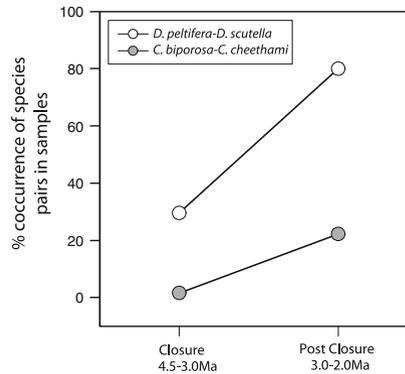
In two species pairs that diverged within, and are extant in the SWC, co-occurrence analysis suggests that they did not co-exist during the first 2 million years after their divergence and during the time of Isthmus emergence and environmental instability in the SWC (4.5–3.0 Ma). Following complete emergence of the Isthmus and environmental stabilization of the SWC (3.5–2.0 Ma) co-existence dramatically increased (Fig. 4).

Discussion

Constraint and plasticity in colony form and life history

Since their origin in the Early Miocene (Cook and Chimonides 1983) *Discoporella* species have attained considerable complexity of forms, including completely in-filled squat

Fig. 4 Percent co-occurrences of species pairs during (4.5–3.0 Ma) and after (3.0–2.0 Ma) closure of the Isthmus of Panama



colonies (e.g. Fig. 1b) in which fragmentation is virtually impossible and which can move easily through sediment if buried (O’Dea 2009); colonies showing ‘colonial budding’, a highly specialized growth form that permits new colonies to be budded when the ‘mother’ colony opts (Marcus and Marcus 1962; Håkansson and Thomsen 2001); and colonies capable of bizarre ‘peripheral fragmentation’, which allows them to be heavily protected while still reproducing clonally (O’Dea et al. 2008; Text-Fig 12). *Cupuladria* species, on the other hand, have yet to approach a similar breadth of reproductive modes and colony forms, thus suggesting constraint in *Cupuladria*, although *C. exfragminis* has evolved a basic form of ‘autofragmentation’ (O’Dea 2006; O’Dea et al. 2008). This constraint may be a function of the inherent differences in colony architecture between the two genera. *Cupuladria* species increase colony strength by multiplying the number of layers of basal kenozoids, whereas *Discoporella* species, which lack basal kenozoids, increase colony strength by simply thickening the amount of basal calcification (see Herrera-Cubilla et al. 2006, 2008).

Although the extremes of morphology and life history that have appeared in *Discoporella* are not apparently tenable for *Cupuladria*, species of *Cupuladria* are more labile within lineages. Thus, during the environmental upheaval that occurred during the emergence of the Isthmus of Panama, *Discoporella* was more likely to diversify than *Cupuladria*, especially into extreme modes of morphology and reproduction, but less likely to phyletically alter life histories in response to change. As a consequence *Discoporella* species were more likely to go extinct (O’Dea and Jackson 2009). Greater extinction rates in *Discoporella* were offset with greater origination rates, resulting in roughly no change in the proportion of *Cupuladria* to *Discoporella* species through the last 10 million years (O’Dea and Jackson 2009).

Several lines of evidence further support the idea that *Cupuladria* species have greater phenotypic plasticity to changing conditions than those of *Discoporella*. (1) Populations of two *Cupuladria* species were observed to alter cloning along environmental gradients in the Recent, but the same pattern did not transpire in any *Discoporella* species (O’Dea et al. 2004). (2) *Cupuladria* but not *Discoporella* autofragments in response to temperature cues (O’Dea 2006). (3) As discussed later, diverging calcification in transisthmian species pairs arose by a decrease in one species and an increase in the other in *Cupuladria*, but only a decrease in *Discoporella* (Fig. 3).

Species isolation within the Caribbean

The isolation of extant geminate (transisthmian) species represents a straightforward model of allopatric speciation (Mayr 1963; Lessios 2008). It is less easy to explain the high origination rates of taxa that occurred at the start of environmental change associated with isthmus closure, that are sympatric today (Budd et al. 1996; Jackson et al. 1996; Smith and Jackson 2009; Quenouille et al. 2010). We propose that increasing physical and environmental heterogeneity $\sim 5\text{--}3$ Ma provided opportunities for localized allopatry (Mayr 1963; Coates and Obando 1996; Jackson et al. 1996) as the narrowing CAS created a complex of archipelagos and rapidly flowing straits connecting the Pacific and Caribbean, as evidenced by the sedimentary settings of the Chagres formation (Collins et al. 1996b), models of paleo-circulation (Haug and Tiedemann 1998; Bartoli et al. 2005; Schneider and Schmittner 2006) and the presence of Pacific-like upwelling penetrating Caribbean environments right up to final closure (O’Dea et al. 2007). It is not difficult to imagine that small populations became isolated by these strong currents passing through the complex archipelago, such as occurs today in the Makassar straits and others formed by the dominating Indonesian Throughflow (Arnaud et al. 1999; Barber et al. 2002; Nuryanto and Kochzius 2009). Indeed, the Indonesian Throughflow may be the best analogy we have today for the processes existing for several millions of years prior to the final emergence of the Isthmus of Panama. Recent studies also suggest similar local isolations and subsequent allopatric speciation of other cheilostome bryozoans in the Aluetian straits (Dick 2008; Dick et al. 2011).

The formation of complex shallow marine habitat has long been considered an important stimulus of speciation for a variety of marine taxa (see Renema et al. 2008) but may be particularly important for groups such as the cupuladriids whose dispersal capacity is severely limited; cupuladriids’ lecithotrophic larvae settle almost immediately after release in close proximity to the reproducing colony (Driscoll et al. 1971; Winston 1988; Cook and Chimonides 1994) and have even been known to settle under the ‘mother’ colony; clonal reproduction is via fragmentation which seeds only locally; calculations of the ‘walking’ ability of colonies demonstrate that colony motility could not be an important dispersal vector; and colonies have never been observed to raft on drift on other, more motile organisms.

Thus, as the island archipelago became more complex, local environmental heterogeneity increased and oceanographic currents intensified, conditions for isolation would have been optimal. The species co-occurrence data demonstrate coeval spatial isolation of SWC species pairs at a local (~ 100 km) level within the oceanographic environment of the Caribbean for 2 million years during the time of final emergence of the Isthmus. After the Isthmus emerged, dramatically increasing co-occurrences of species pairs imply re-unification of already diverged species (Fig. 4). Interestingly, though, changes in colony form in *C. cheethami* and *C. biporosa* as well as *D. peltifera* and *D. scutella* occurred after reconnection of isolated populations, suggesting either (1) niche partitioning of closely related and therefore competing species or (2) further diversification into different microhabitats, e.g. by sediment type and depth, as observed in modern populations (O’Dea 2009), although both hypotheses require further study. The isolation of populations in a geologically short period of time is not unexpected given that rapid speciation has been documented in the fossil record and modern studies across a variety of taxa (e.g. Nehm and Geary 1994; Jackson and Cheetham 1999; Benton and Pearson 2001; Schluter 2001; Gavrillets 2003; Mendelson and Shaw 2005).

Sequential changes in life history and colony form

Species that reduced their level of clonal reproduction survived when food levels fell in the SWC, while species that did not reduce their level of cloning went extinct (O’Dea and Jackson 2009). Our data reveal that those species that survived increased the calcification of their colonies a million years after the oceanographic change and then became squatter (smaller and more conical) a further 1–2 million years later, despite both stronger and squatter colonies being implicated in the protection of the colony from fragmentation and thus in decreased clonality (O’Dea et al. 2004, 2008). This delayed response could reflect the time required for new advantageous traits to evolve and may be expected, given that cloning in many cupuladriids has an ecophenotypic component given that changes in clonality correlate well with environmental change (see previously).

Although increasing calcification of colonies is clearly implicated in reducing rates of cloning (Håkansson and Thomsen 2001; O’Dea 2006, 2009; O’Dea et al. 2004, 2008; O’Dea and Jackson 2009), the role of colony shape is less straightforward. Squatter colony form was originally believed to reduce fragmentation as colonies roll around on the sea floor (O’Dea et al. 2004), but colony shape has since been shown to be intricately related to local habitat conditions, particularly in enabling colonies to move through different sediment types and in preventing overturning in assorted current regimes (O’Dea 2009). We show that the changes in colony height, shape and size that occurred between 3 and 1 Ma coincided with the proliferation of reefs and the establishment of the great coastal heterogeneity characteristic of the modern SWC (O’Dea et al. 2007). Thus, diverging colony morphologies may reflect niche differentiation.

Summary

The emergence of the Isthmus of Panama isolated marine organisms with once continuous populations, as has long been recognized (Mayr 1954; Woodring 1966; Lessios 1981). We propose that there also existed strong isolating mechanisms that caused divergence within the SWC. The first Miocene shoaling event (~8 Ma) did not stimulate habitat heterogeneity and few radiations occurred during this time, but radiations intensified during the Pliocene shoaling (4.5 Ma) that created substantial habitat heterogeneity (Fig. 5). The exceptional cupuladriid fossil record during the second (Late Pliocene) shoaling event demonstrates that new species that originated at this time were more spatially isolated for their first 1–2 million years from their sister species, only to become almost entirely sympatric following final closure (Figs. 4, 5). This strongly suggests that sufficient isolating mechanisms were in place during the tumultuous final uplift of the Isthmus to cause speciation within the SWC (Fig. 5). These isolating mechanisms could have been strong current flow through the straits, as predicted by paleoceanographic modeling and sedimentary evidence. At the time when species were spatially isolated, life histories of species pairs did not diverge but both species pairs changed in response to declining nutrients. Only after closure and re-unification of then-diverged species did colony morphologies in species begin to fully diverge to take advantage of the new heterogeneous post-closure environments (Fig. 5).

Such important information is missed in an exclusively molecular analysis. The cupuladriids demonstrate that by establishing species pairs and then uniting them with exceptionally detailed fossil records across space and through time provides a unique opportunity to observe patterns and processes of speciation and sequence of events in

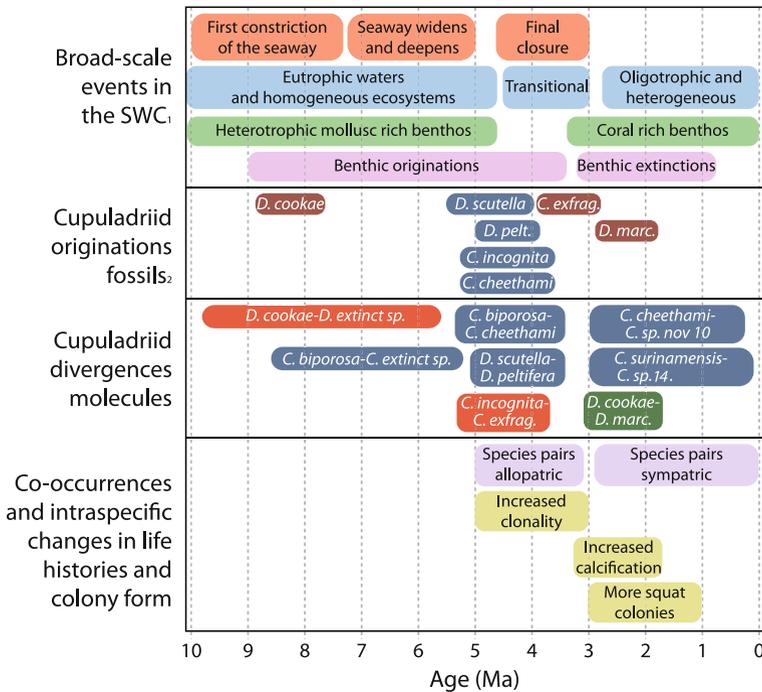


Fig. 5 Sequence of the major abiotic and biotic events in the SWC with the principal episodes in cupuladriid evolution. (1) Duquecaro (1990), Collins et al. (1996a, b), Coates and Obando (1996), Coates et al. (2003, 2004), O’Dea et al. (2007), Jackson et al. (1993), Todd et al. (2002), Smith and Jackson (2009), Johnson et al. (2008), Knowlton and Weigt (1998), Marko (2000). (2) O’Dea and Jackson (2009)

evolution. A more detailed investigation that incorporates morphological phylogenies (of both extinct and extant species) with molecular phylogenies will shed even more light on the evolutionary trajectories of species, providing a more comprehensive picture of how cupuladriids responded to environmental changes related to the emergence of the Isthmus of Panama.

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