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Molecular phylogeny and phylogeography of free-living Bryozoa (Cupuladriidae) from both sides of the Isthmus of Panama

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Abstract

Genetic data were used to identify Recent species of free-living bryozoans (Cupuladriidae) from both sides of the Isthmus of Panama, and to examine their phylogenetic relationships, species richness, and population structures. An approximately 480 bp fragment of the 16S mitochondrial rRNA gene was sequenced from 182 individuals from Panama, the Gulf of Mexico, and El Salvador. Ten haplotype groups (*Cupuladria* 4, 5, and 6; *Discoporella* 1, 2, 3A, 3B, 3C, 7, and 8) were identified. Genetic distances between haplotype groups (3.2–26.5%; K2P + Γ) were 1–2 orders of magnitude greater than within groups (0.1–1.4%). Seven of the haplotype groups represent morphologically distinct species; *Discoporella*s 3A–C appear to be cryptic species. Phylogenetic analyses identified two pairs of transisthmian sister clades. An average divergence rate derived from other taxa suggests that *Cupuladria*s 4 and 5 diverged ≈7 Ma, a *Discoporella* 7 clade diverged from a 3A–C clade ≈11 Ma, and the 3A–C clade radiated ≈6–4 Ma; these events all predated final closure of the isthmus? 3 Ma. The Caribbean side of the isthmus, with 5 species, is only marginally richer in cupuladriids than the Pacific side, with 4, but has greater phylogenetic depth. The Caribbean retains lineages stemming from a New World Miocene radiation that are not represented in the eastern Pacific; extant eastern Pacific cupuladriids share most recent common ancestry with only two of the Caribbean lineages. Species in the eastern Pacific tend to show shallow population structures, with high levels of gene flow between geographically separate populations, whereas Caribbean species tend to show deeper populations structures, with indications of restricted gene flow between Bocas del Toro/Gulf of Mosquitos and Costa Arriba/San Blas. The population structures derive from Pleistocene histories and may be of limited value in interpreting the macroevolutionary pattern, as our results provide no evidence of speciation on either side of the isthmus following closure in the late Pliocene.

1. Introduction

Closure of the Isthmus of Panama in the late Pliocene 3.5–3.1 Ma (Coates and Obando, 1996) provided an unambiguous barrier to gene flow between previously continuous populations of a broad taxonomic range of marine species, resulting in evolutionary divergence of these populations (Lessios, 1998). With special reference to transisthmian taxa, Jordan (1908) coined the term 'geminate species' for pairs of morphologically similar sister taxa on opposite sides of a geographical barrier. Numerous studies have identified geminate species pairs across the Isthmus (e.g., Jordan, 1908; Vermeij, 1978); characterized the degree of morphological difference, molecular divergence, or reproductive isolation between them (e.g., Bermingham and Lessios, 1993; Lessios et al., 1995; Rubinoff and Rubinoff, 1971; Weinberg and Starczak, 1989); correlated degree of reproductive isolation with molecular or morphological divergence (e.g., Knowlton et al., 1993; Lessios, 1981, 1998; Lessios and Weinberg, 1994); and used them to investigate rates of molecular evolution (e.g., Bermingham and Lessios, 1993; Bermingham et al., 1997; Collins, 1996; Cunningham and Collins, 1994; Knowlton and Weigt, 1998; Lessios, 1979).

Closure of the isthmus resulted in radically different near-shore environments in the tropical eastern Pacific versus the Caribbean. Eastern Pacific coastal regions now have a larger tidal amplitude, more variable

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salinity, greater local seasonal upwelling, and more pronounced El Niño/Southern Oscillation climate anomalies than the Caribbean. Correlated biological changes include higher local primary production and planktonic biomass, more predation, and depauperate coral reef development in the tropical eastern Pacific compared to the Caribbean (Glynn, 1972, 1988; Jackson et al., 1996; Jackson and D'Croz, 1997; Rubinoff, 1968; Vermeij, 1978).

Many taxa show differences in diversity between the Caribbean and tropical eastern Pacific. These differences are not all one-way; some taxa, e.g., molluscs (Jones and Hasson, 1985), are more diverse in the eastern Pacific, whereas others, e.g., sponges (Soest, 1994) and corals (Glynn, 1982) are more diverse in the Caribbean. The diversity inequalities are generally attributed to differential speciation and extinction on the two sides of the isthmus (Budd, 1989; Jackson et al., 1993, 1996; Jones and Hasson, 1985; Vermeij, 1978; Vermeij and Petuch, 1986). However, these evolutionary patterns do not explain why taxa responded differently to environmental changes in the two oceans associated with closure of the isthmus. Furthermore, both diversity inequalities and rates of origination and extinction have varied through time, also in a taxon-specific manner. For example, strombinid gastropods were more diverse in the Caribbean than the eastern Pacific until the late Pliocene, when a reversal occurred due to a decline in origination and increase in extinction in the Caribbean, whereas the opposite occurred in the Pacific (Jackson et al., 1993, 1996). In contrast, corals underwent declines in both the eastern Pacific and Caribbean during the late Pliocene, but there was no subsequent Pacific radiation (Budd, 1989; Budd et al., 1996).

How biotas are affected by geographical isolation and environmental change is a question fundamental to ecology, historical biogeography, and phylogeography. The Isthmus of Panama provides a natural laboratory for addressing this question (Cunningham and Collins, 1994; Lessios, 1998), and it makes sense to focus there on taxa for which relevant phylogenetic, phylogeographic, paleontological, and ecological data can be obtained, that is, taxa with a diverse living fauna on both sides as well as an adequate fossil record. Among the most speciose genera in the Neogene fossil record (Coates et al., 1992) along the isthmus are the bryozoan genera *Cupuladria* and *Discoporella* (Fig. 1) of the family Cupuladriidae (Cheetham et al., 1999).

Unlike most other bryozoans, which are sessile, cupuladriids form conical, free-living colonies on particulate bottoms, unattached to the substrate. Distributed in nearshore subtropical and tropical waters worldwide, cupuladriids inhabit sediments ranging from coarse sands to muddy bottoms; cannot tolerate high turbulence or extensive silt deposition; and are most abundant on shelves from 20–70 m (reviewed by Cook, 1965a; Cook and Chimonides, 1983, 1994; McKinney and Jackson, 1989). Cupuladriid species show a remarkable range of morphological and life-history traits, differing in colony size at comparable growth stages; cross-sectional shape and relative thickness of colonies; and patterning and morphology of polymorphic zooids. Furthermore, they can propagate sexually by production of lecithotrophic larvae, or asexually by peripheral budding or regeneration of fragmented colonies. Relative frequencies of these alternative modes of reproduction can be determined for fossil and Recent populations by observation of colony budding patterns.

Fourteen species of fossil cupuladriids are known from Panama (Cheetham et al., 1999). In contrast, the living fauna is poorly known. Only one Recent nominal species has been reported (Hastings, 1929), and Cook (1965b), listed only three nominal species with ranges spanning the Caribbean or Pacific coasts of Panama. The discrepancy between past and present diversity is probably an artifact (Cheetham and Jackson, 2000) due to inadequate sampling of the living fauna, or to misidentification of species as geographical variants of putatively cosmopolitan species. The goals of this study are to use mtDNA sequences to identify Recent cupuladriid species from both sides of the Isthmus of Panama; to compare present species diversity across the isthmus; to reconstruct the phylogeny of the species for insights into the relationship between the Caribbean and eastern Pacific faunas; and to examine the local phylogeographic patterns of the species for signatures of evolutionary processes which might have affected diversity.

2. Materials and methods

2.1. Collecting

Cupuladriids were collected by dredge from the Smithsonian Institution research vessels *Urraca* and *Ziflio*, and in San Blas from a chartered vessel. Specimens were separated from sediment by sieving on board and were preserved in 95% ethanol. In Panama, collecting stations were located in four areas along the Atlantic side of the isthmus (Bocas del Toro, Gulf of Mosquitos, Costa Arriba, and San Blas) and four along the Pacific side (Gulf of Chiriqui, Taboga Island, Las Perlas Islands, and the Darien mainland) (Table 1; see also Fig. 5). Eight *Discoporella* specimens from El Salvador and one *Discoporella* specimen from the vicinity of Panacea, Florida, USA, were also included in the study. Specific locality and depth information for specimens sequenced are available through GenBank accession numbers (Table 2).

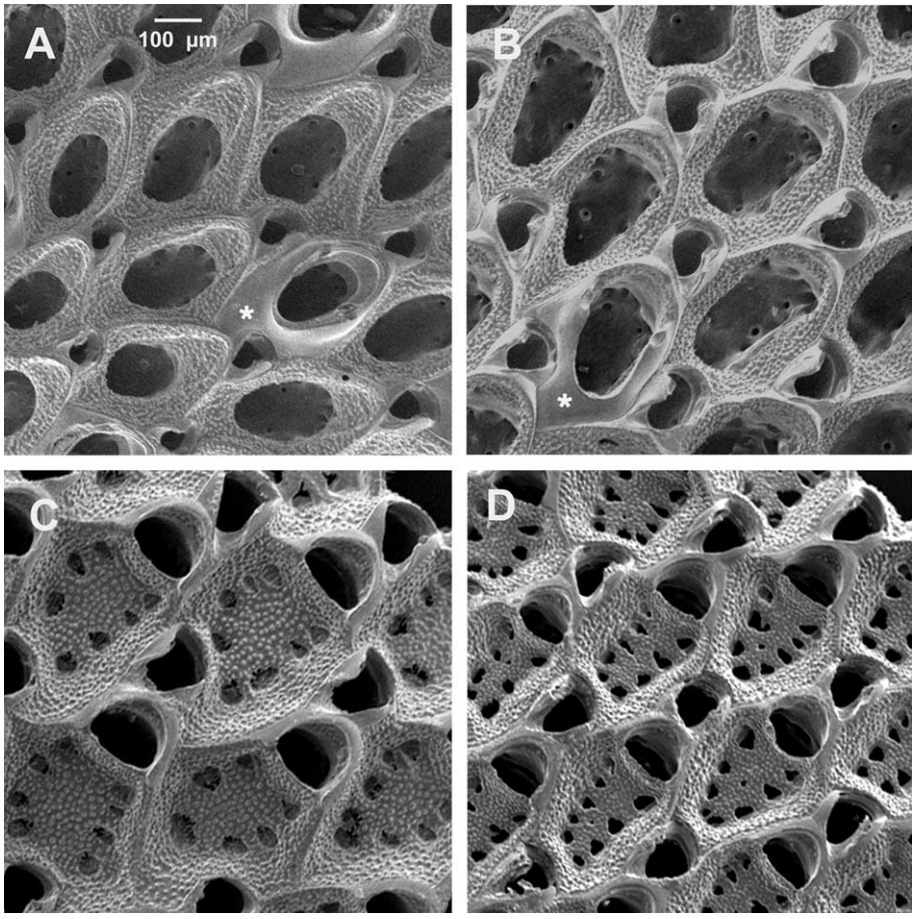

Fig. 1. Representatives of cupuladriid genera *Cupuladria* and *Discoporella*. For each genus, left and right panels contain examples from transisthmian sister clades. (A) *Cupuladria* 4, specimen CA-12, Caribbean; (B) *Cupuladria* 5, specimen TA-1, Pacific; (C) *Discoporella* 7, specimen GM-10, Caribbean; and (D) *Discoporella* 3A, specimen TA-15, Pacific. All panels are to the same scale, and show autozooecia with distal vibracular chambers and (A and B) large vicarious vibracular chambers (asterisks). Note that *Cupuladria* 4 has a tuberculate lamina distal to the opesia, and smaller zooecia than *Cupuladria* 5; *Discoporella* 7 has the opesiules occluded by calcification, and larger zooecia than *Discoporella* 3A.

Table 1
Collecting localities for cupuladriid individuals sequenced in this study

	Latitude range (N)	Longitude range (W)	Depth range (m)	Number of stations[a]
Atlantic (*Caribbean*) *side*				
Bocas del Toro	9°27.70′	82°18.47′	19–30	3
	9°28.45′	82°19.95′		
Gulf of Mosquitos	8°52.70′	81°30.00′	38	1
Costa Arriba	9°35.18′	79°13.04′	19–50	8
	9°40.90′	79°37.22′		
San Blas	9°19.00′	78°17.00′	<20	1
Pacific side				
El Salvador	13°37.40′	90°0.00′	30–35	1
Gulf of Chiriqui	7°49.39′	81°39.87′	9–58	8
	8°00.83′	82°23.48′		
Taboga Island	8°46.70′	79°32.37′	20	1
Las Perlas Islands	8°16.04′	78°19.73′	14–34	8
	8°34.05′	79°19.58′		
Darien mainland	7°40.35′	78°19.70′	71–84	4
	7°49.30′	78°21.20′		

The locality near Panacea, Florida is omitted because coordinates and depth are not available.
[a] Indicates number of stations from which cupuladriids utilized in the study were obtained.

Table 2
Numbers of cupuladriid individuals sequenced by haplotype group and collecting area, number of unique haplotypes, length variation of the 16S fragment, and GenBank accession numbers

Haplotype group	Atlantic					Pacific					*N*	N_h[a]	Length (bp)		GenBank Accession Nos.
	FL	BT	GM	CA	SB	ES	GC	TA	LP	DM			Range	Mode	
Cupuladria															
4	–	10	2	5	4	–	–	–	–	–	21	14	484–486	485	AY123440–60
5	–	–	–	–	–	–	11	3	15	–	29	19	482–483	483	AY123461–89
6	–	4	2	5	–	–	–	–	–	–	11	5	475	475	AY123490–500
Discoporella															
1	1	–	–	–	–	–	–	–	–	–	1	1	478	478	AY123501
2	–	15	–	3	–	–	–	–	–	–	18	6	476	476	AY123502–19
3A	–	–	–	–	–	8	8	11	33	–	60	35	476–478	477	AY123520–79
3B	–	–	–	–	–	–	–	–	–	13	13	7	477	477	AY123580–92
3C	–	–	–	–	–	–	12	–	3	–	15	15	475–478	–	AY123593–607
7	–	–	4	3	–	–	–	–	–	–	7	3	475	475	AY123608–14
8	–	–	3	4	–	–	–	–	–	–	7	7	478-481	478	AY123615–21
Total	1	29	11	20	4	8	31	14	51	13	182	112			

FL, Florida; BT, Bocas del Toro; GM, Gulf of Mosquitos; CA, Costa Arriba; SB, San Blas; ES, El Salvador; GC, Gulf of Chiriqui; TA, Taboga Island; LP, Las Perlas; and DM, Darien Mainland.
[a] Number of unique haplotypes.

2.2. Molecular methods

Prior to DNA extraction, epibionts were scraped and brushed from each colony under a dissecting microscope. Depending on colony size, one-quarter to one-half of the colony was used for DNA extraction; the remainder was dried as a voucher specimen. Samples were ground in liquid nitrogen with a disposable plastic pestle, and total DNA was extracted in urea buffer by the method of Shure et al. (1986). DNA pellets were resuspended in 100 μL water containing 20 μg/mL RNase A.

Two sets of PCR primers were used to amplify a fragment (excluding primers) of the 16S mitochondrial rRNA gene 475–500 bp long, depending on primer set and species. Initially, universal primers 16Sar (5′-CGCCTGTTTATCAAAAACAT) and 16Sbr (5′-CCGGTCTGAACTCAGATC) (Palumbi et al., 1991) were used at an annealing temperature of 48 °C. About 40% of amplicons amplified by these primers, and selected by fragment size for sequencing, proved to be contaminants from symbionts inhabiting the wild-caught bryozoan colonies. Once bona fide sequences were identified from several cupuladriid species, we designed cupuladriid-specific primers CUP16FOR (5′-CGCCTGTTTATCAAAAACATMGCT) and CUP16REV (5′-TCTGAACTCAGATCATGTAAGATA) which totally eliminated amplification of contaminating DNA. PCRs (50 μL) containing 1 μL template solution (unquantitated; total DNA extracted from a colony portion resuspended in 100 μL water), 2 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol each primer, and 1.5 U AmpliTaq DNA polymerase in 1× Buffer A (Perkin–Elmer) were carried out in an MJ Research PTC-200 Peltier thermal cycler. PCR conditions for the cupuladriid-specific primers were 95 °C, 2 min; 40 cycles (95 °C, 30 s; 54 °C, 30 s; 74 °C, 90 s); 74 °C, 5 min.

PCR products were ligated into pGEM T-easy vector (Promega), and plasmids transformed into JM108 competent cells according to the manufacturer's instructions. A cloning strategy was employed initially because of the problem of amplification of contaminants by the universal primers. Inserts were amplified from plasmids by PCR using M13 (−20) and M13 rev primers; conditions were as above but in 25 μL volumes, with 35 cycles and 56 °C annealing temperature. Excess primers were removed and dNTPs dephosphorylated by incubation of 20 μL PCR product (sequencing template) solution for 2 h at 37 °C with 8 U exonuclease I and 1.5 U shrimp alkaline phosphatase, followed by 10 min at 80 °C. Late in the study, we found that amplification products from the cupuladriid-specific primers could be sequenced directly. All sequencing reactions contained 4 μL of template solution, 2 μL (3.2 pmol) SP6 or T7 primer, 2 μL Half-Term buffer (Genpak) and 2 μL dRhodamine Mix (Perkin–Elmer); reaction conditions were 25 cycles (96 °C, 15 s; 1 °C/s to 50 °C; 50 °C, 1 s; 1 °C/s to 60 °C; 60 °C, 4 min). Excess dRhodamine Mix was removed using Centri-Sep columns (Princeton Separations) with Sephadex G-50 Fine (Sigma S-5897). All sequences were determined from both directions with an ABI 377 XL automated sequencer. ABI Prism Sequencing Analysis 3.3 and Sequencher 3.1 programs were used to record and edit sequence data. All sequences have been deposited in GenBank as population sets by haplotype group (see Table 2 for accession numbers).

2.3. Phylogenetic and population analyses

Celleporella sp. Cal-7 (GenBank AF156278) was used as the outgroup. The sister group to the Cupuladriidae among cheilostome bryozoans is unknown. *Celleporella* emerged as the closest outgroup to a monophyletic cupuladriid ingroup in maximum parsimony searches including available 16S sequences from representatives of a range of anascan, cribrimorph, and ascophoran genera (including also *Thalamoporella*, *Scrupocellaria*, *Copidozoum*, *Cellaria*, *Cribrilina*, *Figularia*, *Celleporina*, *Microporella*, *Schizomavella*, and *Schizoporella*). Although *Celleporella* belongs to a different nominal suborder (Ascophora) than the Cupuladriidae (Anasca), there is evidence suggesting the Ascophora may not be monophyletic, and that the sister group to the family Hippothoidae, which includes *Celleporella*, may lie among the Anasca or Cribrimorpha (Dick et al., 2000). The basal branching order and general topology of the cupuladriid ingroup remains quite stable regardless which anascan or cribrimorph taxon is used as outgroup; if all phylogenetic information had been lost through saturation effects, this would not be the case.

All unique cupuladriid haplotypes were aligned for phylogenetic analysis using Sequencher 3.1 and adjusted manually. The outgroup was then aligned with the cupuladriids manually. The alignment had 498 characters, of which 11 gap-rich positions were discarded. The data set thus comprised 487 positions, of which 217 (45%) were constant, 98 (20%) parsimony-uninformative, and 172 (35%) parsimony-informative. The sequences were A–T rich (mean A + T, 67%), but did not significantly differ in base composition ($\chi^2 = 91.79$, df = 336, $p = 1.0$)

Phylogenetic analyses were conducted with PAUP* 4.0b10 (Swofford, 2000). For maximum parsimony (MP), 1000 heuristic search replicates were performed using random addition sequence. Uninformative characters were excluded, informative characters were weighted equally and unordered, and gaps were treated as missing. Non-parametric bootstrap values (Felsenstein, 1985) were determined by heuristic analysis of 500 pseudosamples of the original data set of informative characters, with replacement. Decay indices of nodes (Bremer, 1988; Donoghue et al., 1992) were determined using the filter trees option in PAUP*. A neighbor-joining (NJ) tree was constructed using log-determinant distances. For ML analysis, the data set was reduced from 113 to 68 taxa by pruning 45 sequences from haplotype groups *Discoporella* 3A and 3B and *Cupuladria* 4 and 5. A NJ tree from the reduced data set was used to estimate parameters for models of nucleotide substitution and as a starting tree for branch-swapping in heuristic maximum likelihood (ML) searches. Modeltest version 3.06 (Posada and Crandall, 1998) indicated that the GTR model with a discrete approximation of the gamma distribution (GTR + Γ, 4 rate categories, shape parameter 0.55; Rodriguez et al., 1990; Yang, 1994) provided the best fit to the data.

A variety of corrected genetic distance measures relevant to transisthmian divergences have been reported in the literature for the 16S gene, the most prevalent of which have been based on the Kimura 2-parameter model (K2P, Kimura, 1980), and K2P with a discrete approximation of the Γ distribution (K2P + Γ). Unless otherwise noted, distance measures given in the text refer to K2P + Γ. To examine variation in rates of molecular evolution, a ML tree was determined containing a single sequence from each haplotype group (the most common haplotype or the consensus sequence). This tree had the same topology as the ML tree obtained from the pruned data set (Fig. 4). Tests of relative-rates between clades, based on K2P distances, were performed using RRTree version 1.1 (Robinson-Rechavi and Huchon, 2000).

As indicators of population structure and history (Grant and Bowen, 1998; Nei, 1987), we determined indices of haplotype and nucleotide diversity for each haplotype group. Haplotype diversity (h) was calculated manually as $h = 1 - \Sigma f_i^2$, where f_i is the frequency of the ith haplotype. Nucleotide diversity (π) was calculated with Matrix version 2.0 software (Posada, 2001) as $\pi = \Sigma f_i f_j p_{ij}$, where f represents haplotype frequency and p_{ij} is the sequence divergence between the ith and jth haplotypes.

To estimate relative degrees of gene flow between geographically separate populations within haplotype lineages, we calculated F_{ST} statistics with Arlequin version 2.0 (Schneider et al., 2000). This software takes an analysis of molecular variance (AMOVA) approach to obtain an estimate of F_{ST} identical to Wier and Cockerham's (1984) θ_W. We included in the F_{ST} analyses populations with sample sizes of three or more individuals, and quantified DNA differences using Tamura and Nei (1993) distances. The probability of obtaining the observed F_{ST} values by chance was estimated from 10,100 random reshufflings of haplotypes between populations, with significant values considered to be those for which less than 5% of the random reshufflings produced larger values.

3. Results

3.1. Phylogeny and geographical distributions

Partial 16S sequences were obtained from 182 cupuladriid colonies; among these sequences, 112 unique haplotypes were identified (Table 2). The comparable region of the 16S gene amplified by the two primer sets ranges in length from 475–486 bp, with minor length variation within some haplotype groups (Table 2).

Nine monophyletic haplotype groups are evident in the NJ tree (*Cupuladria* 4, 5, and 6, and *Discoporella* 2, 3A, 3B, 3C, 7, and 8; Fig. 2). *Discoporella* 1, for which monophyly was not assessed, is considered an additional haplotype group because it shows genetic distances $\geqslant 6.67\%$ from the others (Table 3). All but one of the monophyletic groups identified in the NJ tree are also monophyletic in the strict consensus of 1111 MP trees (Fig. 3). *Discoporella* 3A appears as an unresolved polytomy with the *Discoporella* 3B + C clade and comprises a paraphyletic group. Bootstrap and Bremer support for the monophyletic haplotype groups is generally high, with *Cupuladria* 4 the least well supported. In the ML tree from the pruned data set (Fig. 4), the same monophyletic haplotype groups appear, with *Discoporella* 3A again emerging as unresolved and paraphyletic.

The NJ, MP, and ML trees show a similar general topology among the haplotype groups. *Cupuladria*s 4

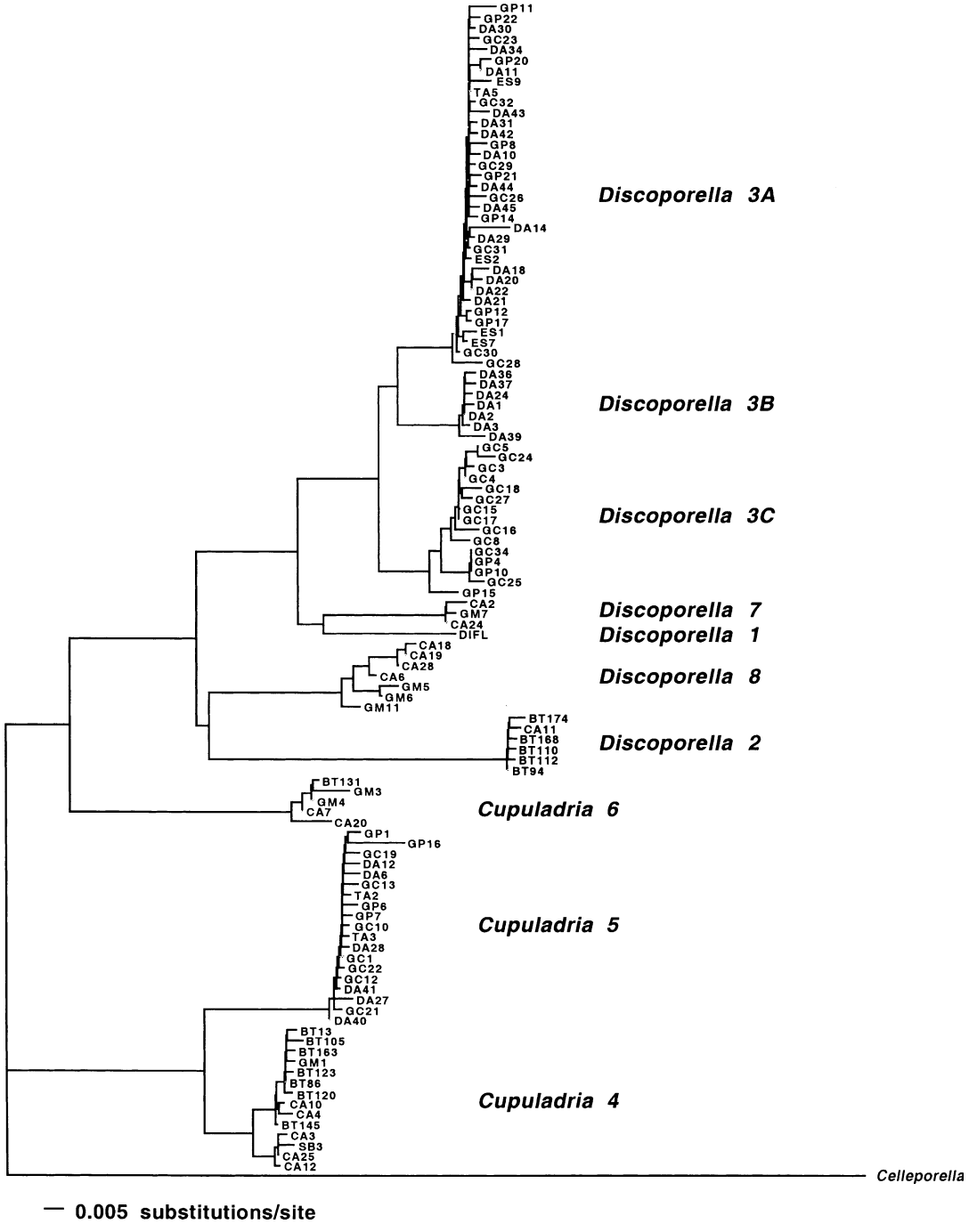

Fig. 2. Neighbor-joining (NJ) tree based on log determinant/paralinear distances, including 112 unique cupuladriid haplotypes, with *Celleporella* sp. Cal-2 (GenBank AF156278) as the outgroup. Monophyletic haplotype groups are designated to the right of the tree.

Table 3
Average genetic distances (%) in pairwise comparisons of sequences between and within haplotype groups

Taxon	*Cell*	*Cup*4	*Cup*5	*Cup*6	*Dis*2	*Dis*8	*Dis*1	*Dis*7	*Dis*3A	*Dis*3B	*Dis*3C
Celleporella	–	30.38	30.88	33.78	37.21	33.83	35.26	36.57	34.72	35.80	34.48
Cupuladria 4	**40.26**	**0.86**	5.40	15.08	20.63	17.43	19.93	19.02	19.21	19.06	19.05
Cupuladria 5	**41.17**	**5.70**	**0.46**	17.34	21.16	19.81	21.58	20.06	20.91	20.57	20.51
Cupuladria 6	**46.08**	**17.27**	**20.32**	**0.73**	16.76	13.85	16.00	15.85	16.45	16.99	17.67
Discoporella 2	**53.88**	**25.03**	**25.80**	**19.54**	**0.14**	12.87	14.77	14.69	14.92	16.46	16.48
Discoporella 8	**46.74**	**20.45**	**23.82**	**15.69**	**13.59**	**1.44**	11.83	11.61	10.89	11.45	12.86
Discoporella 1	**49.42**	**24.03**	**26.48**	**18.49**	**17.06**	**13.22**	–	6.27	7.27	7.92	8.93
Discoporella 7	**52.27**	**22.70**	**24.26**	**18.29**	**16.94**	**13.31**	**6.67**	**0.18**	6.94	8.10	9.04
Discoporella 3A	**48.33**	**22.94**	**25.43**	**19.09**	**17.20**	**12.34**	**7.81**	**7.52**	**0.37**	3.08	4.12
Discoporella 3B	**50.44**	**22.77**	**24.94**	**19.83**	**19.25**	**13.63**	**8.55**	**8.85**	**3.18**	**0.26**	4.25
Discoporella 3C	**47.86**	**22.74**	**24.84**	**20.44**	**19.24**	**15.78**	**9.74**	**9.96**	**4.31**	**4.46**	**1.15**

Above diagonal: distances estimated by Kimura's (1980) 2-parameter model (K2P). Below diagonal, in bold: distances estimated by K2P with a discrete approximation of the Γ distribution (K2P + Γ). Along diagonal: within-group distances, K2P above and K2P + Γ below. Positions containing gaps were ignored in pairwise comparisons.

and 5 are sister clades, basal to other cupuladriids. *Cupuladria* 6 is a sister clade to all *Discoporella*s. However, there are some differences among the analyses in the topology of the *Discoporella* clade. The NJ tree (Fig. 2) shows *Discoporella*s 2 + 8 as a sister group to other *Discoporella*s, whereas this relationship is unresolved in the MP (Fig. 3) and ML (Fig. 4) trees. A *Discoporella* 1 + 7 clade comprises a sister group to the *Discoporella* 3A–C clade in the NJ and MP trees, although the 1 + 7 grouping has low bootstrap and Bremer support. In contrast, *Discoporella* 7 alone is the sister group to the *Discoporella* 3A–C clade in the ML tree. Finally, both the MP and ML trees show *Discoporella* 3A as a paraphyletic group basal to a *Discoporella* 3B + C clade, though the *Discoporella* 3B + C grouping has low bootstrap and Bremer support. The NJ analysis shows an alternate grouping of *Discoporella* 3A with *Discoporella* 3B.

The genetic distances between haplotype groups are one to two orders of magnitude greater than the distances within groups (Table 3). Average between-group distance is 17.0%, (range 3.18% for *Discoporella* 3A–B to 26.48% for *Cupuladria* 5—*Discoporella* 1), whereas average within-group distance is 0.62% (range 0.14% for *Discoporella* 2–1.44% for *Discoporella* 8).

Fig. 5 shows the known local geographical ranges of the haplotype groups along the Isthmus of Panama, inferred from the observed distributions given in Table 2. *Discoporella* 3A was also found in El Salvador. Most of the lineages are co-distributed with others on the same coast. However, there is a clear disjunction between *Discoporella* 3B, which was detected only along the Darien mainland, and *Discoporella*s 3A and 3C, which co-occur in the Gulfs of Panama and Chiriqui.

3.2. Phylogeographic patterns of the haplotype groups

Parsimony networks for the nine Central American haplotype groups are presented in Fig. 6. Five haplotype groups (*Discoporella*s 2, 3A, 3B, and 7; *Cupuladria* 5) show a "star phylogeny" pattern (Avise, 2000), each characterized by a dominant ancestral-like haplotype, with other haplotypes divergent from the dominant form by 1–4 mutational steps. In all cases, the dominant haplotype is distributed across the geographical range sampled. The dominant haplotype of *Discoporella* 3A, for example, comprises 43% of the sequences of that group and occurs in three geographically separate areas: the Gulf of Panama (Las Perlas and Taboga I.), the Gulf of Chiriqui, and El Salvador. Haplotype groups showing this pattern have low (<0.50) to high (0.68–0.84) haplotype diversities, and low (<0.50%) nucleotide diversities (Table 4).

Four haplotype groups (*Discoporella*s 3C and 8; *Cupuladria*s 4 and 6; Fig. 6) lack a clearly dominant haplotype, show greater mutational distances among some haplotypes, and exhibit high haplotype and nucleotide diversities (Table 4). *Cupuladria* 4 has a "dumbbell" pattern (Fig. 6), with two star phylogeny structures separated by a longer branch. Most of the *Discoporella* 3C

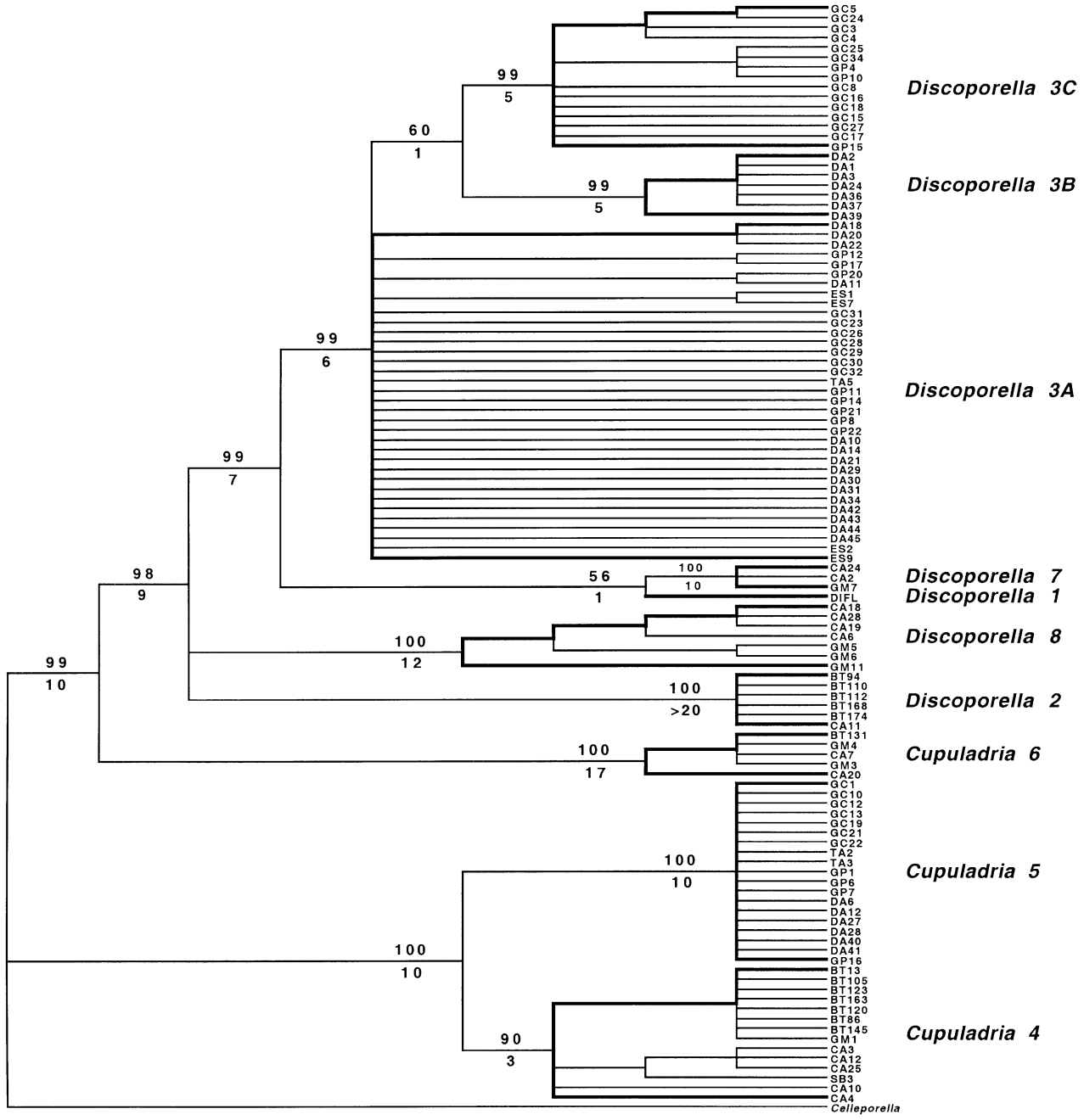

Fig. 3. Strict consensus of 1111 equally optimal maximum parsimony (MP) trees including 112 unique cupuladriid haplotypes and the outgroup. Length = 405 steps; CI = 0.6519; RI = 0.9654; informative characters only. Numbers above and below internal branches show boostrap values >50% and Bremer decay indices, respectively. Monophyletic haplotype groups identified in the NJ tree (Fig. 2) are outlined in bold and designated to the right of the tree.

haplotypes in the Gulf of Chiriqui comprise a clade descendent from a common ancestral haplotype, whereas the three haplotypes detected in Las Perlas belong to two divergent lineages. However, two haplotypes related to those in Las Perlas also occur in the Gulf of Chiriqui. The pattern of *Discoporella* 8 is similar to that of *Discoporella* 3C, though the sample size is smaller. The pattern of *Cupuladria* 6 is complex, showing both common as well as rarer, divergent haplotypes.

3.3. Degree of genetic exchange between populations

Haplotype groups (*Discoporella*s 2, 3A, 7; *Cupuladria* 5) which show a star phylogeny phylogeographic pattern (Fig. 6) and low intra-clade variation also exhibit low F_{ST} values (−0.09 to 0.08) between geographically separate populations (Table 5). Although most of these values are non-significant, with greater values likely to be obtained by chance, they are consistent with the

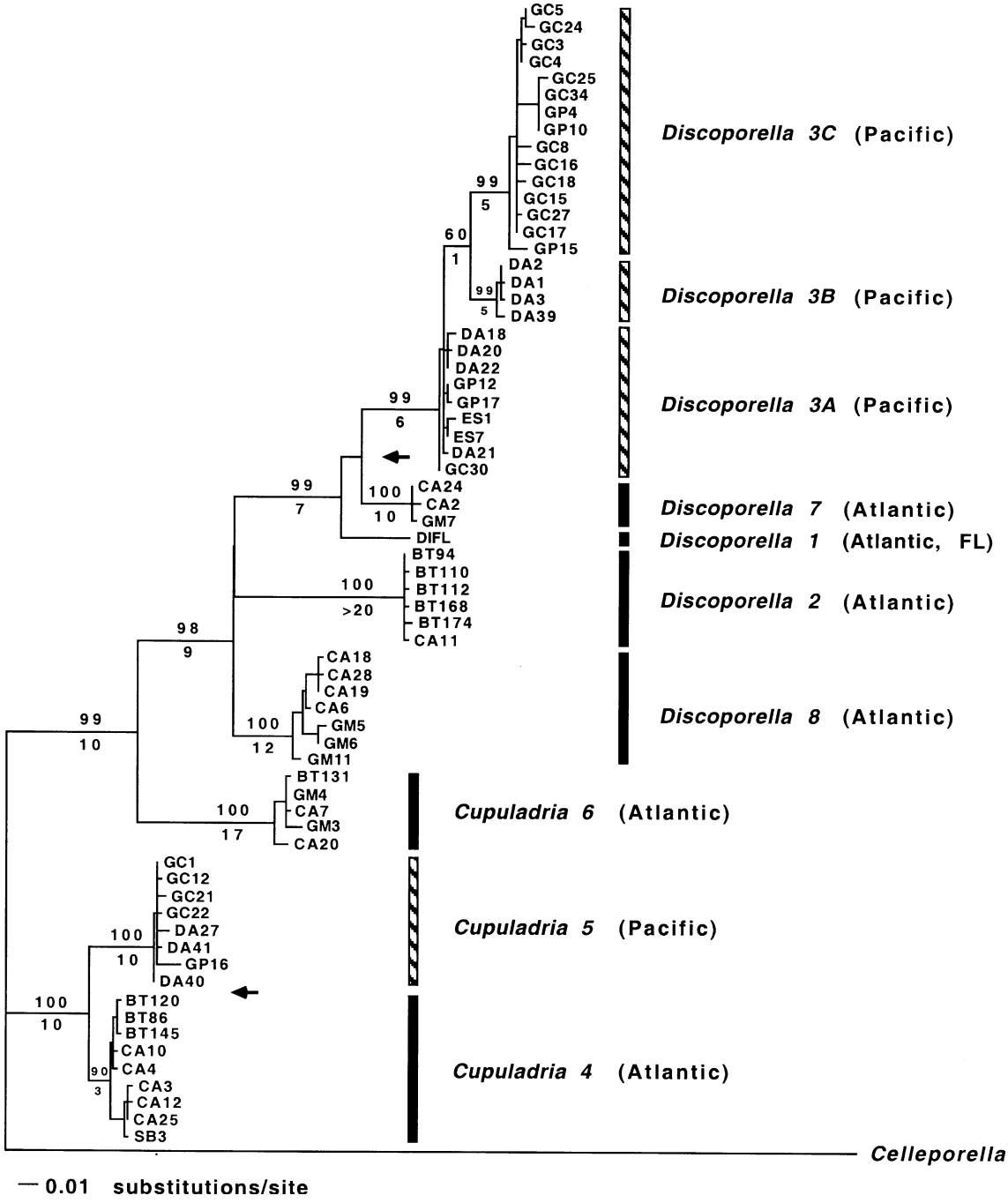

Fig. 4. Maximum likelihood (ML) phylogeny from the pruned data set, including 67 unique cupuladriid haplotypes and the outgroup. Numbers above and below branches repeat the bootstrap values and decay indices, respectively, derived from the MP analyis (Fig. 3). −Ln likelihood = 2921.737; GTR + Γ; shape parameter $\alpha = 0.55$. Hatched bars: haplotype groups from the Pacific side of the isthmus. Solid bars: haplotype groups from the Atlantic side. Arrows indicate transisthmian sister clades.

phylogeographic pattern and suggest high levels of gene flow between populations. Furthermore, except for *Discoporella* 2, the highest values (not shown) in the null distributions derived by random reshuffling still indicate high levels of gene flow.

High and significant F_{ST} values (Table 5) for *Cupuladria* 4 indicate restricted gene flow between Bocas del Toro and Costa Arriba, and even greater isolation of the Bocas del Toro and San Blas populations. A lower, though non-significant, F_{ST} value as well as a shared haplotype indicates gene flow occurs between Costa Arriba and San Blas.

Cupuladria 6 and *Discoporella* 8, which both exhibit complex phylogeographic patterns with considerable divergences among haplotypes, also exhibit population structuring along the Caribbean coast. A significant F_{ST}

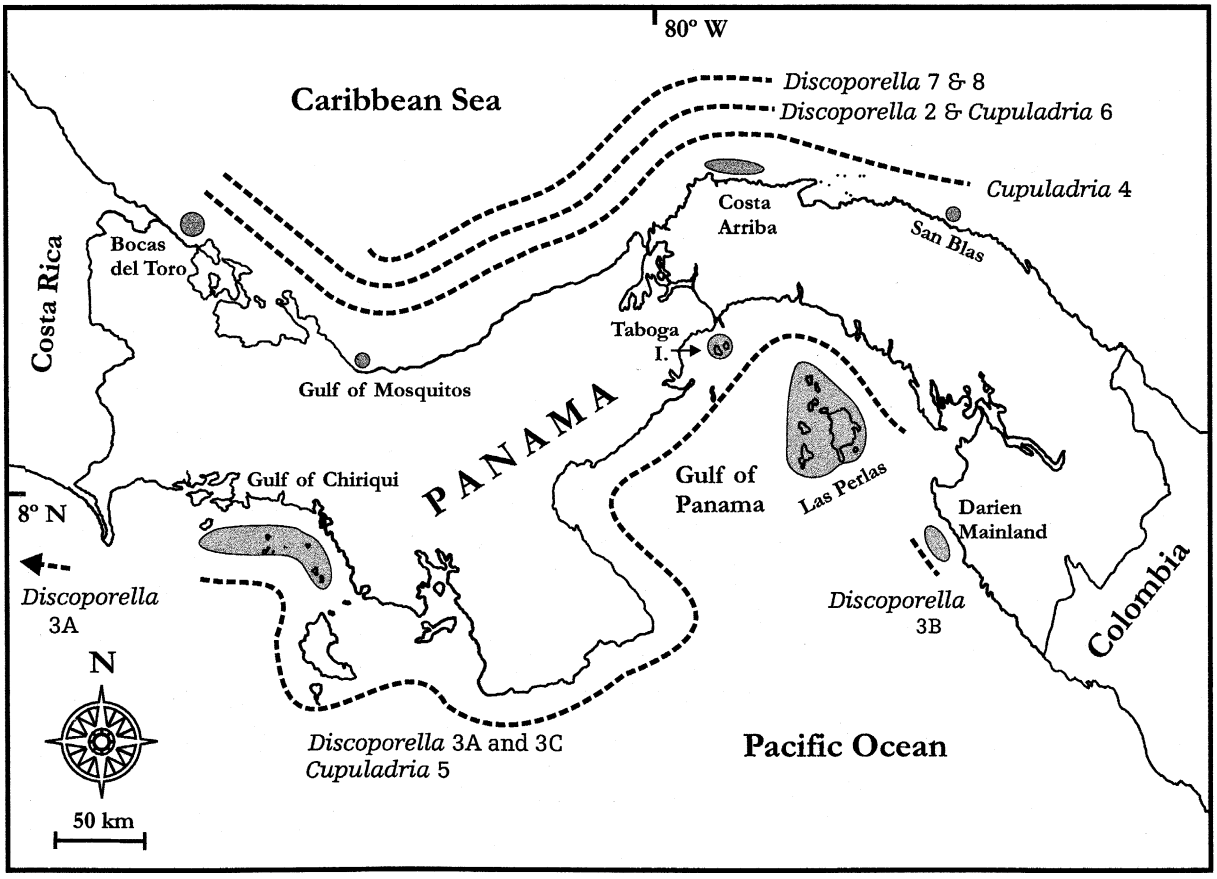

Fig. 5. Map of Panama showing collecting areas (shaded). Dashed lines indicate known local distributions of cupuladriid haplotype groups. Arrow at bottom left indicates records of *Discoporella* 3A from El Salvador. *Discoporella* 1 from Florida is not shown.

value of 0.39 indicates restricted gene flow between Bocas del Toro and Costa Arriba populations of *Cupuladria* 6. The F_{ST} value from a comparison between Gulf of Mosquitos and Costa Arriba populations of *Discoporella* 8 is high, but non-significant.

Discoporella 3C shows a phylogeographic pattern of divergent lineages that are sympatric over at least part of their range. The F_{ST} value of 0.23 (Table 5) between the populations in the Gulf of Chiriqui and the Gulf of Panama is significant, and is equivalent to an exchange of 1.7 females per generation at genetic equilibrium, which means the populations are not genetically isolated.

4. Discussion

4.1. Species status of the haplotype groups

Six haplotype groups (*Cupuladria*s 4, 5, 6; *Discoporella*s 2, 7, 8) clearly represent species-level phylogenetic lineages. These comprise well-supported monophyletic groups (Figs. 2–4), and are separated by average genetic distances ranging from 5.7–26.5% (Table 3). *Discoporella* 1 from Florida, for which only one individual was sequenced and thus monophyly not examined, nonetheless shows a minimum distance from other haplotype groups of 6.67%, which is greater than that, for example, between the transisthmian geminate clades *Cupuladria*s 4 and 5. Although there is no absolute scale by which genetic distance can indicate biological species status (Harrison, 1991; Knowlton, 2000), these haplotype groups also show diagnostic morphological differences (see Fig. 1 for examples). A treatment of morphology is beyond scope of this paper, and will be presented elsewhere.

It is unclear whether *Discoporella*s 3A–C represent divergent intraspecific lineages versus cryptic species. No diagnostic morphological differences are obvious among them, and *Discoporella* 3A appears in the MP and ML trees as a paraphyletic group basal to *Discoporella*s 3B and 3C. However, the average genetic distances among these groups (3.18–4.46%) indicate divergences occurred prior to closure of the isthmus (see following section), which supports their status as cryptic species

Furthermore, there are indications of ecological differences among *Discoporella*s 3A–C. *Discoporella* 3B, found only along the Darien mainland, is disjunct from *Discoporella* 3A and 3C (Fig. 5). The former was found at depths ranging from 71–84 m, whereas the latter were

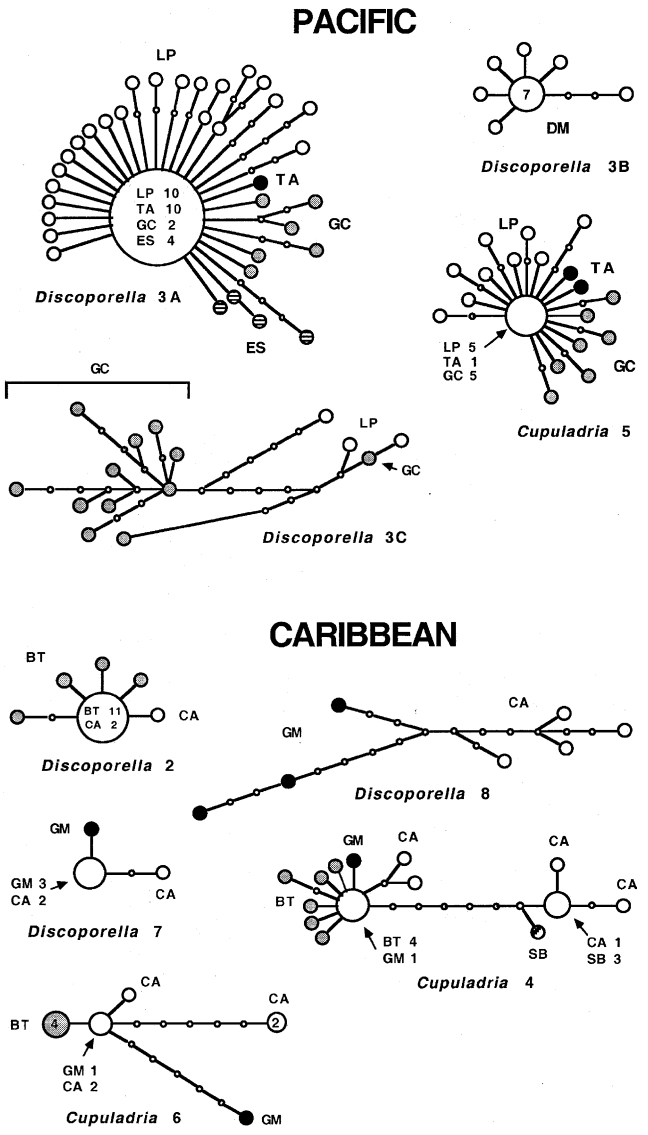

Fig. 6. Parsimony networks of 16S mtDNA haplotypes, by ocean and haplotype group. Circles represent unique haplotypes. Most are of unit size, indicating haplotypes detected once. Size of larger circles is proportional to number of identical haplotypes; smallest unfilled circles indicate intermediate haplotypes not observed. Each branch between two haplotypes indicates one base change, including insertions/deletions. Different filling of unit circles indicates geographical locality, labeled BT (Bocas del Toro), CA (Costa Arriba), DM (Darien mainland), ES (El Salvador), GC (Gulf of Chiriqui), GM (Gulf of Mosquitos), LP (Las Perlas), SB (San Blas), or TA (Taboga I.).

shallower, ranging from 23–58 m. Although *Discoporella*s 3A and 3C are sympatric with one another in the Gulfs of Panama and Chiriqui, and sometimes occur together in the same dredge haul, they show differences in relative abundance between the two regions. *Discoporella* 3A was 5.5 times more abundant than 3C in samples from the Gulf of Panama, and individuals sequenced from around Taboga Island ($N = 11$) and from the Bay of San Telmo in Las Perlas Islands ($N = 17$) were all 3A. Conversely, *Discoporella* 3C was 1.5 times more abundant than 3A in the samples from the Gulf of Chiriqui, and individuals from one locality, Uva I. ($N = 4$), were all 3C. These apparent differences in relative abundance might be related to different ecological preferences between the two lineages, as the Gulfs of Panama and Chiriqui are environmentally somewhat different (Glynn, 1972). Sequences from additional loci, morphometric analyses, and information on reproductive isolation and ecology will be necessary to clarify the species status of these *Discoporella*s.

The paraphyly of the *Discoporella* 3A haplotype group is difficult to interpret. Failure to resolve a 3A clade in the MP and ML phylogenies (Figs. 3 and 4), and low bootstrap and Bremer support for the 3B + 3C grouping, may indicate these three lineages radiated rapidly relative to time since radiation, with the 3A polytomy an artifact of the shallow population structure of this haplotype group. However, the longer branch lengths of the 3B and 3C lineages compared to 3A are difficult to account for except through unequal rates of substitution. A relative-rate test (RRTree version 1.1, Robinson-Rechavi and Huchon, 2000) based on K2P distances and using *Discoporella* 7 as the reference taxon does indicate significantly different rates between the 3A and the 3B + 3C lineages ($p = 0.029$).

Among the haplotype groups, we have identified only *Cupuladria* 6 with a previously described species, *C. surinamensis* Cadée (1975), known from the Guyana Shelf off the northeast coast of South America. The transisthmian geminate clades *Cupuladria*s 4 and 5 are both similar to *C. biporosa* described from the Caribbean Miocene by Canu and Bassler (1923), redefined by Cook and Chimonides (1994), and their taxonomic status needs to be evaluated. Some of the *Discoporella* species identified by our study are certainly new. Either they were not present in collections examined by previous workers, or they were identified as *Discoporella umbellata* subsp. *depressa* (Conrad, 1841), previously considered to be distributed in tropical and subtropical waters of both the western Atlantic and the eastern Pacific (e.g., Cook, 1965a,b; Winston, 1982).

4.2. Timing of transisthmian separations

The phylogenies (Figs. 2–4) reveal two instances of sister clades occupying opposite sides of the Isthmus of Panama: Atlantic *Cupuladria* 4—Pacific *Cupuladria* 5, and Atlantic *Discoporella* 7 (or 1 + 7)—Pacific *Discoporella*s 3A–C. The average genetic distance between *Cupuladria*s 4 and 5 is 5.70% (K2P + Γ; Table 3), whereas that between *Discoporella* 7 (+1) and the 3A–C clade is higher (range 7.52–9.96%). If one assumes that the smaller distance, between *Cupuladria*s 4 and 5, represents divergence following final closure of the isthmus 3.1 Ma, then this gives an estimated rate of divergence of 1.8%/my.

Table 4
Indices of haplotype and nucleotide diversity for cupuladriid haplotype groups

Haplotype group	Frequency of common haplotype (%)	Haplotype diversity (h)	Nucleotide diversity (π%)	Side of isthmus
Cupuladria				
4	24	0.76	0.83	Atlantic
5	38	0.84	0.46	Pacific
6	36	0.74	0.71	Atlantic
Discoporella				
2	72	0.46	0.14	Atlantic
3A	43	0.80	0.37	Pacific
3B	54	0.68	0.26	Pacific
3C	–	0.93	1.12	Pacific
7	71	0.45	0.18	Atlantic
8	–	0.86	1.39	Atlantic

Discoporella 1 is omitted because only one individual was sequenced. For *Discoporella*s 3C and 8, all sequences were unique.

Table 5
F_{ST} statistics comparing populations within haplotype groups

Cupuladria 4			
Overall $F_{ST} = 0.62$*			
	Bocas del Toro (10)	Costa Arriba	
Costa Arriba (5)	0.53*		
San Blas (4)	0.82*	0.21	
Cupuladria 5			
Overall $F_{ST} = -0.03$			
	Gulf of Chiriqui (11)	Las Perlas	
Las Perlas (15)	−0.01		
Taboga I. (3)	−0.03	−0.09	
Cupuladria 6			
	Costa Arriba (5)		
Bocas del Toro (4)	0.39*		
Discoporella 2			
	Costa Arriba (3)		
Bocas del Toro (15)	−0.00		
Discoporella 3A			
Overall $F_{ST} = 0.00$			
	El Salvador (8)	G. Chiriqui	Taboga I.
Gulf of Chiriqui (8)	0.02*		
Taboga I. (11)	0.08*	0.04*	
Las Perlas (33)	0.02	0.01	−0.04
Discoporella 3C			
	Las Perlas (3)		
Gulf of Chiriqui (12)	0.23*		
Discoporella 7			
	Costa Arriba (3)		
Gulf of Mosquitos (4)	0.05		
Discoporella 8			
	Costa Arriba (3)		
Gulf of Mosquitos (4)	0.47		

Sample sizes are indicated in parentheses. Comparisons in which less than 5% of 10,100 random reshufflings produced larger values are indicated with asterisks.

This rate is roughly two to three times higher than previous estimates for the 16S gene derived from transisthmian geminates calibrated on a closure of the isthmus ≈3 Ma. Previous estimates include 0.9%/my (fiddler crabs; Sturmbauer et al., 1996; K2P + Γ), 1.0%/my (average for two geminate species pairs in the fish genus *Centropomus*; Tringali et al., 1999; K2P + Γ), 0.53%/my (porcelain crabs; Stillman and Reeb, 2001;

K2P + Γ), 0.65%/my (two sets of geminate clades of grapsid crabs; Schubart et al., 1998; K2P), and 0.67%/my (mangrove-associated chthamaloid barnacles; Wares, 2001; GTR + Γ). Although not obtained from transisthmian calibrations, similar rates have been observed for molluscs: 0.54–0.96 %/my (*Mytilus*; Rawson and Hilbish, 1995) and 0.57%/my (zebra mussels; Stepien et al., 1999; K2P).

Although it is possible the rate of divergence is actually higher in cupladriids, we consider this unlikely, given that previous estimates from disparate phyla (arthropods, molluscs, chordates) fall within a narrow range, approximately 0.5–1.0%/my. The alternative is that the rate in cupuladriids is similar to that in other taxa, and that *Cupuladria*s 4 and 5 diverged before final closure of the isthmus. An average rate based on the three previous transisthmian studies that use a K2P + Γ correction is 0.8%/my. Applying this rate to the average divergence of 5.70% (Table 3) between *Cupuladria*s 4 and 5 gives an estimated time of separation of ≈7 Ma (Late Miocene). Similar calculations indicate that separation of the Pacific *Discoporella* 3A–C lineage from Caribbean *Discoporella* 7 (average distance 8.78%) occurred ≈11 Ma (early late Miocene), and that divergences among *Discoporella*s 3A–C (distances 3.18–4.46%; Table 3) occurred ≈6–4 Ma (late Miocene-early Pliocene).

These estimates of divergence times are rough indicators at best, based on assumptions that the sister clades we have identified are true geminates, that the timing of final closure ≈3 Ma is accurate, that an average rate from other taxa holds for cupuladriids, and that the cupuladriid 16S sequences evolved in a clock-like manner. A likelihood ratio test comparing the log likelihood value from the ML tree to that obtained under the assumption of a molecular clock shows significant deviation from a constant rate ($\delta = 160$, $df = 67$, $p < 0.005$). However, relative rate tests (RRTree version 1.1, Robinson-Rechavi and Huchon, 2000) based on K2P distances indicate that the only significant ($p < 0.05$) rate variation occurs in the *Discoporella* 3A–C clade and between *Cupuladria*s 4 and 5. We report no confidence limits for the calculated divergence times because they are not based on a clock calibrated from independently derived estimates of cupuladriid divergence times (Hillis et al., 1996).

Closure of the Isthmus of Panama ≈3 Ma was the culmination of a series of transitions that began 12 my earlier, and involved progression from a deep ocean seaway, to formation of an archipelago, to increasingly isolated sedimentary basins along the developing barrier (Coates, 1997; Coates and Obando, 1996; Duque-Caro, 1990). There is evidence from the snapping shrimp genus *Alpheus* that, contrary to previous assumptions, transisthmian sister species were separated throughout this long formation of the isthmus, with relatively few separations occurring at closure (Knowlton and Weigt, 1998; Knowlton et al., 1993).

One explanation for gene flow restriction prior to closure is that populations inhabiting deeper water became isolated from one another as shoal depths decreased. However, this alone is not satisfactory to explain so many pre-closure divergences. The depths of *Cupuladria*s 4 and 5 in our collections range from 17–50 m, whereas channels across the forming isthmus during the late Miocene (7–6.3 Ma) may have been too deep (<150 m; Duque-Caro, 1990) to restrict gene flow if the ancestral population had a depth range similar to extant populations. But, as the isthmus rose, changes also occurred in sea surface circulation patterns and in the environments of the emergent basins (Collins et al., 1996; Duque-Caro, 1990; Jackson and Budd, 1996), both of which could have restricted gene flow. Knowlton and Weigt (1998) found divergences between most transisthmian pairs of *Alpheus* fell within a 3–9 (3–10 Ma; their Fig. 2) Ma range, with a pulse of divergences at about 4.5 Ma coinciding with oceanographic measures of basin isolation. The cupuladriid results are similar to these findings.

4.3. Relationship of the eastern Pacific and Caribbean cupuladriid faunas

The Caribbean side of the isthmus, with 5 species identified, appears only marginally richer in cupuladriids than the Pacific side, with 4 (assuming *Discoporella*s 3A–C are cryptic species). Local assemblages are also more diverse on the Caribbean side. The highest richness in a single dredge haul was 4 species at Gulf of Mosquitos (*Cupuladria*s 4 and 6, *Discoporella*s 2 and 8), and the highest richness in a single sampling area was 5 species at Costa Arriba (Table 2). On the Pacific side, the highest richness encountered per haul or per sampling area was 3 species (*Cupuladria* 5; *Discoporella*s 3A and 3C).

The small difference in raw diversity across the isthmus masks a greater difference in phylogenetic depth. The cupuladriids detected in the eastern Pacific originated in the late Miocene to early Pliocene and share most recent common ancestry with only two of the Caribbean lineages, *Cupuladria* 4 and *Discoporella* 7 (Fig. 4). This is consistent with the conclusion of Cook and Chimonides (1983) that Recent cupuladriids from southern California, western Mexico, and the Galapagos are "a relic population of Gulf [of Mexico] species." In contrast, most of the Caribbean species represent lineages descendent from an earlier Miocene radiation that no longer occur in the eastern Pacific.

The Gulf of Mexico was an early center of cupuladriid diversity in the New World. Before the Miocene, several groups of free-living bryozoans unrelated to cupuladriids (Lunulitidae, Otionellidae, and Selenariidae; Bock and Cook, 1999) occurred in the Gulf. These went extinct in the Gulf and Caribbean at the end

of the Oligocene and were completely and non-competitively replaced there in the Miocene by cupuladriids via dispersal, probably from the eastern Atlantic (Cook and Chimonides, 1983; Lagaaij, 1963). A record of Miocene *Discoporella* from Chile (Philippi, 1887 in Cook and Chimonides, 1983) indicates that cupuladriids spread widely along the eastern Pacific coast after reaching the Gulf.

Our results suggest that earlier Miocene *Discoporella* lineages went extinct in the eastern Pacific, followed by the subsequent Pacific radiation in late Miocene-early Pliocene of the *Discoporella* 3A–C clade. Our study provides no evidence for more recent cupuladriid speciation in either the Caribbean or eastern Pacific. However, the divergent *Cupuladria* 4 lineages that are geographically separate between Bocas del Toro and San Blas (Fig. 6) perhaps represent incipient allopatric species that have come into secondary contact. Distance between the dominant haplotypes found at these respective localities is 1.27%, indicative of a Pleistocene divergence ≈1.6 Ma. Both lineages occur at Costa Arriba, which may indicate hybridization is occurring there; data from nuclear loci would be informative in this regard.

4.4. Comparison of eastern Pacific and Caribbean population structures

Cupuladriid populations in the eastern Pacific appear to be less structured than those in the Caribbean. Three of the four haplotype groups in the Pacific show star phylogeny structures (Fig. 6), with high haplotype and low nucleotide diversities (Table 4), indicative of populations that have expanded after a period of low effective population size (Grant and Bowen, 1998). Low F_{ST} values indicate considerable gene flow among these populations. *Discoporella* 3A, for example, shows no genetic structuring between populations over a length of coast spanning approximately 1600 km from the Gulf of Panama to El Salvador. Although *Discoporella* 3C shows deeper structure than the other Pacific haplotype groups, there is enough gene flow between populations in Gulfs of Chiriqui and Panama that they cannot be considered isolated.

In contrast, only two of the Caribbean cupuladriid species show star phylogeny structures (*Discoporella*s 2 and 7; Fig. 6). Both haplotype and nucleotide diversities of these are low (Table 4), attributable to relatively recent colonization events or periodic regionwide bottlenecks; examination of metapopulation structure is necessary to distinguish between these alternatives. Three Caribbean species show deeper population structure, with high haplotype and nucleotide diversities (Table 4), indicative of larger, more stable populations over evolutionary time (*Cupuladria* 6 and *Discoporella* 8) or secondary contact between allopatrically divergent lineages (*Cupuladria* 4) (Grant and Bowen, 1998). For these species, F_{ST} statistics (Table 5) indicate limited gene flow between populations at Bocas del Toro/Gulf of Mosquitos and those at Costa Arriba/San Blas.

A higher frequency of shallow population structures in the eastern Pacific compared to the Caribbean might indicate that Quaternary climatic fluctuations had differential effects across the isthmus, causing more extreme or more frequent fluctuations in population sizes in the Pacific. However, such a conclusion is tenuous. The number of species examined was small, and the apparent difference might be stochastic. Furthermore, examination of metapopulation structures could considerably alter the perceived patterns. For example, sampling of *Cupuladria* 4 only at Bocas del Toro and Gulf of Mosquitos (Fig. 6) would have revealed a star phylogeny pattern, whereas broader sampling revealed deeper population structure.

Finally, the intraspecific phylogeographic patterns we observed may be of limited value in explaining the macroevolutionary pattern. The genetic data provide no evidence of speciation on either side of the isthmus following closure. These patterns likely reflect Pleistocene histories, and Pleistocene climatic oscillations in general appear to have had little impact on speciation or extinction rates in most marine taxa (Jablonski and Sepkoski, 1996; Jackson, 1994; Knowlton, 2000; Roy et al., 1996), although there are exceptions (Jackson et al., 1996; Knowlton, 2000). Species which survived the filter of a late Pliocene turnover responded to Pleistocene oscillations by latitudinal changes in abundances or geographical range boundaries, which may even have increased their evolutionary stability by preventing long-term isolation and genetic divergence (Roy et al., 1996).

Use of 16S mtDNA sequences has allowed us to identify Recent cupuladriid species and putative cryptic species with some confidence. Genetically identified voucher specimens provide a starting point for morphometric analyses to establish the continuity of Recent with fossil lineages, and to examine the correlation between molecular divergence at the DNA level and morphological change. Confidence in the taxonomy of cupuladriids through time will in turn permit calibration of a molecular clock to date divergences for which the fossils are uninformative. Caribbean and eastern Pacific cupuladriid faunas have followed different historical paths to arrive at a seemingly trivial difference in absolute Recent species diversity, although additional sampling throughout the Caribbean may change this conclusion. Integration of information on the ecologies of Recent cupuladriid species with other types of data has the potential to provide a unique insight into how biotas are affected by geographical isolation and environmental change.

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