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Flying shells: historical dispersal of marine snails across Central America

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The geological rise of the Central American Isthmus separated the Pacific and the Atlantic oceans about 3 Ma, creating a formidable barrier to dispersal for marine species. However, similar to Simpson's proposal that terrestrial species can 'win sweepstakes routes'—whereby highly improbable dispersal events result in colonization across geographical barriers—marine species may also breach land barriers given enough time. To test this hypothesis, we asked whether intertidal marine snails have crossed Central America to successfully establish in new ocean basins. We used a mitochondrial DNA genetic comparison of sister snails (*Cerithideopsis* spp.) separated by the rise of the Isthmus. Genetic variation in these snails revealed evidence of at least two successful dispersal events between the Pacific and the Atlantic after the final closure of the Isthmus. A combination of ancestral area analyses and molecular dating techniques indicated that dispersal from the Pacific to the Atlantic occurred about 750 000 years ago and that dispersal in the opposite direction occurred about 72 000 years ago. The geographical distribution of haplotypes and published field evidence further suggest that migratory shorebirds transported the snails across Central America at the Isthmus of Tehuantepec in southern Mexico. Migratory birds could disperse other intertidal invertebrates this way, suggesting the Central American Isthmus may not be as impassable for marine species as previously assumed.

Keywords: geminate species; *Cerithideopsis*; *Cerithidea*; Isthmus of Panama; Central America

1. INTRODUCTION

The separation of populations by geographical barriers and dispersal across those barriers are two major opposing forces determining species formation and distribution. The final closure of the Central American Seaway by the rise of the Isthmus of Panama divided two tropical oceans [1] and had profound consequences for marine biota [2,3]. Marine organisms were separated by a land barrier and adapted to very different environments, promoting genetic divergence and allopatric speciation [3]. Although several studies have considered the divergence of populations separated by the Isthmus [2,3], natural dispersal of marine organisms across Central America has received little attention.

In 1940, Simpson [4] proposed that terrestrial species can win highly improbable 'sweepstakes' and disperse across ocean barriers. He later noted that 'a possible dispersal event, however improbable. . .becomes probable if enough time elapses' [5, p. 171]. We hypothesized that some marine species may be particularly prone to winning such sweepstakes dispersal across Central America by hitchhiking on birds. Some intertidal snails are abundant in migratory shorebird feeding habitats, can adhere to feathers, bills and legs of birds [6–9], and can survive after being ingested [10,11]. Because many species of shorebirds regularly cross Central America at both the Isthmus of Panama and the Isthmus of Tehuantepec [12–16], we suspected that such snails could be transported across Central America by this route.

The Pacific horn snail, *Cerithideopsis californica* (=*Cerithideopsis mazatlanica* and *Cerithideopsis valida*) and the Atlantic horn snail, *Cerithideopsis pliculosa* constitute a pair of 'geminate species [17]' formed following separation by the geological formation of the Isthmus of Panama [18]. These species (formerly in the genus *Cerithidea* [19]) are common and often very abundant in intertidal mangrove and mudflat habitats [19], which are major foraging areas for migratory shorebirds. Further, the snails are known to survive ingestion by birds [10], suggesting that they may be particularly prone to avian transport. When documenting that *C. californica* and *C. pliculosa* were geminate species, Miura *et al.* [18] provided preliminary evidence that these snails may have historically dispersed between the Pacific and the Atlantic coasts. Here, we provide a more rigorous testing of this possibility, broadly sampling snails along both coasts and evaluating genetic evidence

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which indicates that these marine snails have indeed crossed the Central American land barrier.

2. MATERIAL AND METHODS

(a) *Sample collections*

We collected *C. californica* and *C. pliculosa* from 29 populations across 30° of latitude along both coasts of North and Central America (electronic supplementary material, table S1). Snails were either frozen or preserved in 95 per cent EtOH. All samples were stored at −20°C in the laboratory for molecular analyses.

We isolated DNA using a modified CTAB procedure [20]. A small piece of tissue from the foot of each snail was homogenized in a solution of 300 ml 2x CTAB and 10 mg ml^{-1} proteinase K, and incubated at 60°C for approximately 1 h, extracted once with phenol/chloroform (v : v, 1 : 1) and precipitated with two volumes of ethanol. The DNA pellets were briefly washed in 75 per cent ethanol, air-dried for approximately 30 min and dissolved in 50 ml of H_2O.

(b) *DNA sequencing*

We analysed mitochondrial DNA encoding the cytochrome oxidase *c* subunit I (CO1) gene to identify major mitochondrial clades and their phylogeographic patterns. We also analysed the 16S ribosomal RNA gene (16S), 12S ribosomal RNA gene (12S), cytochrome *b* gene (Cytb) and NADH dehydrogenase subunit 6 gene (ND6) for a single individual from each major clade to estimate times of divergence events. The primer pairs used in this study are listed in electronic supplementary material, table S2. PCR reactions ran for 35 cycles under the following conditions: denaturing at 94°C for 60 s, annealing at 50°C for 60 s (45°C for CO1) and extension at 72°C for 90 s. The 35 cycles were preceded by an initial denaturing at 94°C for 1 min, followed by a final extension of 72°C for 7 min. The PCR products of the samples were purified and sequenced using an automated sequencer (ABI 3130xl). Sequences analysed in this study were deposited in GenBank (accession nos HQ724852–HQ725015).

(c) *Estimation of phylogenetic relationships and genetic parameters*

Sequences were aligned by ClustalW, implemented in BioEdit [21] for further phylogenetic analyses. Phylogenetic trees based on the CO1 sequences were constructed using maximum-likelihood analysis (ML) and Bayesian inference (BI). We used the Akaike Information Criterion to determine the best model using Modeltest [22] (the sequences were not partitioned for codon positions). The GTR + I + G model was selected for the CO1 gene. The most closely related congener, *Cerithideopsis pulchra*, was selected for an outgroup based on a higher level phylogeny of *Cerithideopsis* [18]. The ML analysis was conducted using PHYML [23] using a BioNJ starting tree and rate parameter optimization. Node robustness was assessed using non-parametric bootstrapping and 1000 replicates. The BI tree was obtained using MrBayes [24]. The dataset was run for four million generations with a sample frequency of 100. The first 25 per cent of trees were discarded, such that only 30 001 trees were accepted. Because the software automatically analysed the data in two independent runs, a total of 60 002 trees were analysed to estimate phylogenetic relationships and posterior probability value of each clade. Convergence between the two runs was tested by examining the potential scale reduction factors.

We used a molecular clock on the five mitochondrial genes (CO1, 16S, 12S, CytB and ND6; approx. 3000 bp total) to estimate divergence time of the major genetic clades. We calibrated the molecular clocks with the final closure of the Panamanian Isthmus at 3.1–2.8 Myr ago [3]. We analysed only mitochondrial DNA variation because the available nuclear genes are generally too conserved to allow comparison between the geminate snails [18]. While amplified fragment length polymorphism (AFLP) markers would be ideal to evaluate shallow divergences [25], mitochondrial DNA provided sufficient resolution for tracking potential dispersal events across the Isthmus owing to its rapid rate of sequence divergence [26] and absence of recombination [27]. A likelihood ratio test rejected ($p < 0.01$) the hypothesis of a molecular clock [28]. Thus, dates of divergence were inferred using a relaxed molecular clock, following the uncorrelated relaxed lognormal clock implemented in BEAST v. 1.5.4 [29]. A uniform prior distribution was used for the split of the clades assumed to be separated by the rise of the Isthmus. The Yule speciation model was used as a tree prior. The best evolutionary model was determined using Modeltest [22], which selected HKY for the 12S gene, HKY + I for 16S gene, K3Puf + I for ND6 gene and HKY + I + G for the CO1 and Cytb genes. The combined dataset was partitioned among genes. We used gene-specific models and unlinked all parameters among genes. The analysis was run for 10 million generations, sampled every thousand steps and the first thousand samples were discarded as burn-in. To check for convergence and to visualize the results, we used Tracer v. 1.4.1 and FigTree v. 1.2.3 [29]. Ideally, fossil calibration would provide another independent measure for our dating analysis [30], but fossil records for *Cerithideopsis* are not available.

We obtained CO1 haplotypes network trees using maximum parsimony and TCS 1.21 [31].

We tested the monophyly of snails from the Pacific or snails from the Atlantic using both the Shimodaira–Hasegawa (SH) test [32] and the approximately unbiased (AU) test [33] in CONSEL [34]. We constrained the Pacific and the Atlantic snails to be monophyletic, respectively, and tested it against the unconstrained (best) tree.

We used three types of ancestral area analyses to identify ancestral areas for the major clades: (i) dispersal–vicariance analysis [35], (ii) ancestral area analysis (AAA [36]), and (iii) weighted ancestral area analysis [37]. All AAAs used a cladogram based on the phylogeny shown in figure 1.

We compared CO1 haplotype variation within and among populations using statistics from analysis of molecular variance using Arlequin v. 3.0 [38].

3. RESULTS AND DISCUSSION

We found high mitochondrial DNA CO1 genetic variation along both coasts (figure 1). Snails were separated into two major phylogenetic clades (clades A and B), both composed of several genetically well-separated subgroups. First, we identified the genetic divergence associated with the closure of the Central American Seaway [3]. The two major clades A and B are characterized by 8.5 per cent K2P distance and 29.7 per cent Ks (silent site) distance (point 1, figure 1). This distance corresponds with the CO1 genetic distances that Lessios [3]

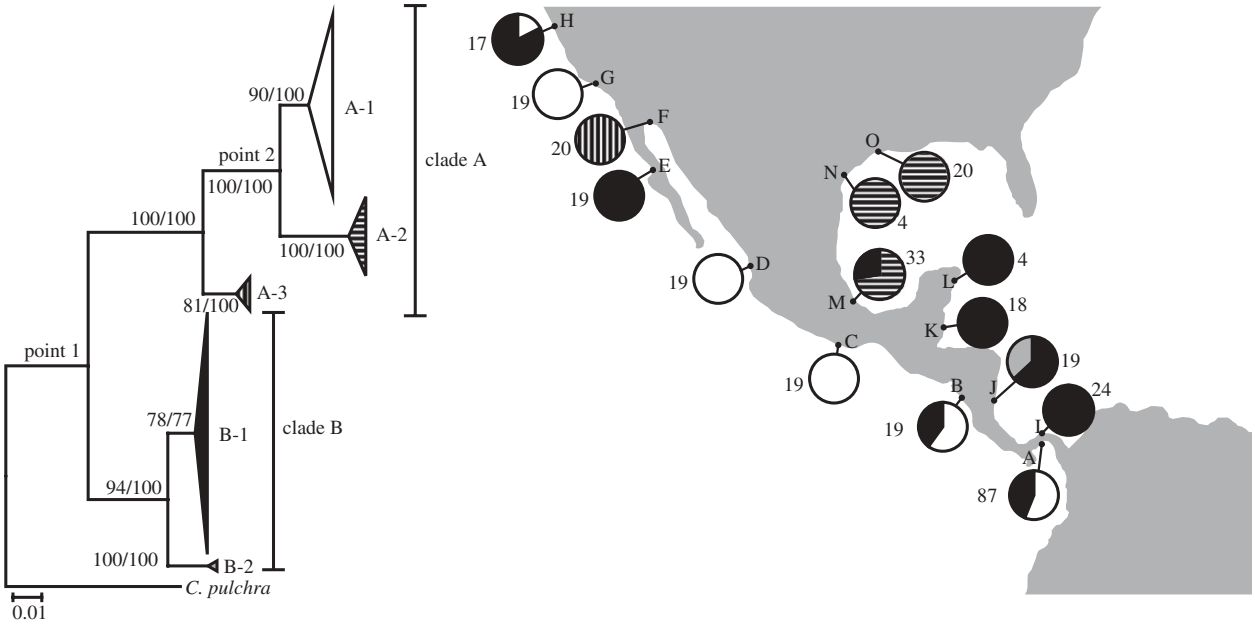

Figure 1. Molecular phylogeny and geographical distribution of the intertidal snails, *Cerithideopsis californica* and *C. pliculosa*. A maximum-likelihood tree was constructed based on 873 bp of the CO1 gene. The major clades are categorized as clades A, B and the detailed subclades are within. Numbers near nodes are the support values for the clade from the different analyses (ML/BI). The scale bar represents the phylogenetic distances expressed as units of expected nucleotide substitutions per site. Phylogenetic relationships within the clades are shown in electronic supplementary material, figure S1. The distributions of the major genetic clades are shown at the right side of the figure. Numbers near the geographical points indicate sample size. Letters indicate sampling sites (see electronic supplementary material, table S1).

reported for four other gastropod geminate species pairs separated by the closure of the Seaway (average K2P distance 7.4–9.2% and average Ks distance 23.5–30.3%) and with what Miura *et al.* [18] report for this geminate pair. Interestingly, both clades A and B contain individuals from both coasts. This is obvious in figure 1 and, further, monophyly of the snails from the Pacific or from the Atlantic is rejected by both the SH and AU tests ($p < 0.01$). A divergence within clade A (point 2) separates Pacific and Atlantic individuals (subclades A-1 and A-2; figure 1). Genetic divergence (K2P = 4.0%, Ks = 12.6%) at this point was less than half the divergence characterizing point 1 or the distances separating other geminate gastropods [3]. Similarly, the Pacific and the Atlantic snails in subclade B-1 exhibited a small divergence (K2P = 0.9%, Ks = 2.4%; see electronic supplementary material, figure S1). These results suggest that separation by the rise of the Isthmus of Panama produced the major genetic clades, A and B, and that after the final closure of the Seaway there were at least two dispersal events between the Pacific and the Atlantic.

We used the phylogenetic patterns of each major clade to reconstruct ancestral distributions and infer directionality of the cross land barrier dispersals. All three historical biogeographic analyses identified the Pacific as the ancestral area of clade A, and the Atlantic as the ancestral area of clade B (table 1). These methods implicitly employ phylogenetic diversity to infer the ancestral area, as ancestral populations probably have higher diversity than more recently colonized sites. Indeed, clade A has two highly diverged subclades in the Pacific (A-1 and A-3), while only a single subclade was found in the Atlantic (A-2). Similarly, clade B has two genetically distinct subclades in the Atlantic (B-1 and B-2), while only one of those (B-1) appears in the Pacific. Importantly, snails from the upper Gulf of California form a unique subclade (A-3, figure 1), owing to the isolating influence driven by the Baja California Peninsula [39], and the presence of this unique and old lineage in the Pacific further indicates a Pacific origin of clade A. Similarly, the basal subclade B-2 was found only along the Atlantic Coast of Nicaragua (figure 1), suggesting the Atlantic origin of clade B. These results further support that the rise of the Isthmus of Panama isolated clade A in the Pacific and clade B in the Atlantic. These groups diversified to several subclades within each ocean after the Isthmus formed. Subsequently, individuals from clade A dispersed from the Pacific to the Atlantic, forming subclade A-2, and individuals of subclade B-1 dispersed more recently from the Atlantic to the Pacific, creating its current distribution.

Table 1. The results of the ancestral area analyses. (Ancestral areas were estimated by dispersal–vicariance analysis (DIVA), the ancestral area analysis (AAA), and the weighted ancestral area analysis (WAAA). All results indicate the Pacific origin of clade A and the Atlantic origin of clade B.)

clade	ocean	DIVA	AAA (G/L)	WAAA (PI)
clade A	Pacific	O	2.00	3.00
	Atlantic	—	0.50	0.33
clade B	Pacific	—	0.50	0.33
	Atlantic	O	2.00	3.00

We calibrated the molecular clock for the five mitochondrial genes assuming that the divergence at point 1 corresponds to the final closure of the Central American Seaway at 3.1–2.8 Myr ago [3]. Analyses indicated that dispersal from the Pacific to the Atlantic occurred about

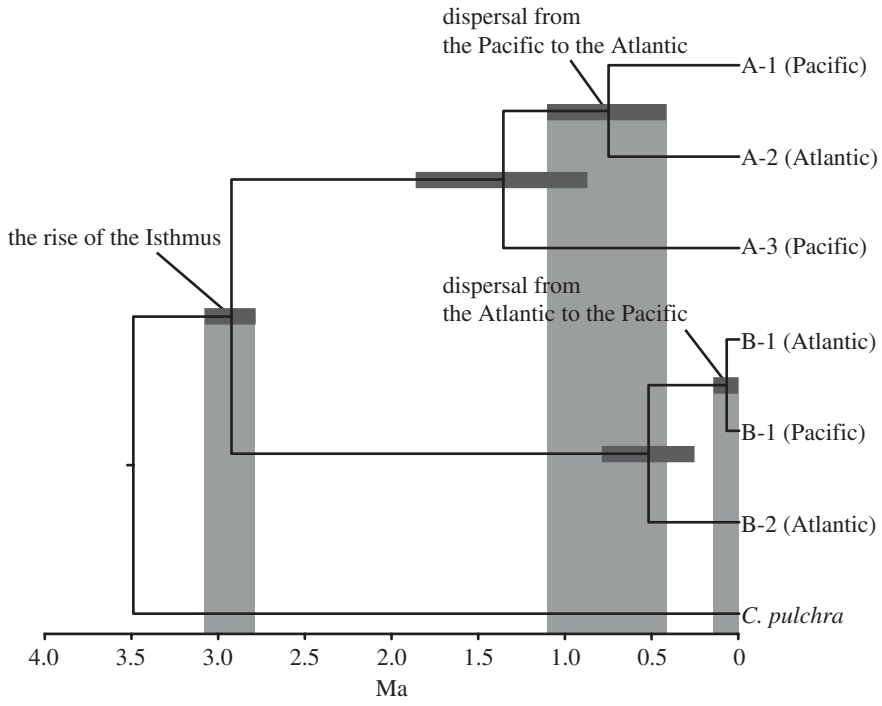

Figure 2. Divergence time estimates for the major clades in *C. californica* and *C. pliculosa* based on the CO1, 16S, 12S, Cytb and ND6 genes (totally 2952 bp). Horizontal bars represent the upper and lower interval bounds for 95% of the highest posterior densities (HPDs). Each gene tree is shown in electronic supplementary material, figure S2.

750 000 years ago (430 000–1 100 000 years ago: 95% highest posterior density (HPD); figure 2) and that dispersal from the Atlantic to the Pacific occurred about 72 000 years ago (13 000–147 000 years ago: 95% HPD, figure 2).

Until the 1914 breach of the Panama Isthmus by the Panama Canal, coastal organisms were completely separated by land. Dispersal over the Central American land barrier is the most likely explanation for our results. It is implausible that the snails dispersed around the North American arctic or the southern tip of South America (approx. 20 000 km) because the geographical range of *Cerithideopsis* does not extend beyond warm temperate regions [40] and the snails are either direct developers or have short-lived larvae [41]. Additionally, the absence of identical mitochondrial haplotypes on both sides of the Isthmus of Panama despite extensive sampling (electronic supplementary material, table S1) suggests that *Cerithideopsis* has not dispersed through the approx. 100 year old Panama Canal, as have some other marine organisms [42,43]. Temporary connections between the Pacific and the Atlantic Oceans about 1.9 Ma [44] are thought to have facilitated the post-Isthmian dispersal of some marine species [45]. However, our dates (0.02–0.14 and 0.43–1.1 Myr ago) for dispersal events are too late for this and much earlier than would be predicted for human-mediated dispersal.

We posit that transport by migrating shorebirds is the most parsimonious explanation for the bidirectional dispersal of *Cerithideopsis* across Central America. *Cerithideopsis* snails live in habitats used by millions of shorebirds. These shorebirds regularly cross Central America over two migration flyways: the Isthmus of Tehuantepec in southern Mexico [12–14,16], and the Isthmus of Panama [15]. Further, birds can transport small invertebrates [7]. Small, juvenile *Cerithideopsis* snails could become attached to bird feathers or appendages via their sticky mucus. Additionally, several species of shorebirds ingest *Cerithideopsis* and the snails can survive. For example, Sousa [10] found approximately 30 per cent survivorship of *Cerithideopsis* snails in regurgitation pellets of the willet (*Tringa semipalmata*). Regurgitation can occur days to weeks after feeding [46], which is sufficient time to cross either the Tehuantepec or Panama *trans*-Isthmian flyways (200 and 70 km wide, respectively).

To identify the most likely locations of the over-land dispersals, we constructed haplotype networks. Concerning the dispersal of clade A from the Pacific to the Atlantic, all encountered members of subclade A2 were in the Gulf of Mexico (figure 1). Further, the network tree shows that A2 haplotypes in Galveston, USA, are most closely related to Pacific A1 haplotypes (figure 3*a*). This suggests dispersal occurred in the Gulf of Mexico, across the Isthmus of Tehuantepec rather than over the narrower Isthmus of Panama. The lack of geographical structure for Pacific A1 precludes elucidating a specific Pacific source location for the colonization (table 2). Concerning the dispersal from the Atlantic to the Pacific, the network tree of the subclade B1 shows that all Pacific haplotypes were derived from a haplotype currently in Mandinga, Mexico (figure 3*b*). This suggests that snails dispersed south across the Isthmus of Tehuantepec from the Gulf of Mexico to the Pacific. Here, the data suggest a source for the Atlantic to the Pacific dispersal (Mandinga) but the lack of genetic structure in Pacific B1 populations precludes pinpointing a colonization location (table 2). The relative lack of genetic structure in Pacific *Cerithideopsis* populations (both A1 and B1) may be owing to greater planktonic dispersal ability of Pacific snails at low latitudes [41]. Nevertheless, the available data suggest that both dispersal events occurred across the Isthmus of Tehuantepec.

Contemplating the distribution of species across the continents, Simpson argued that given enough time,

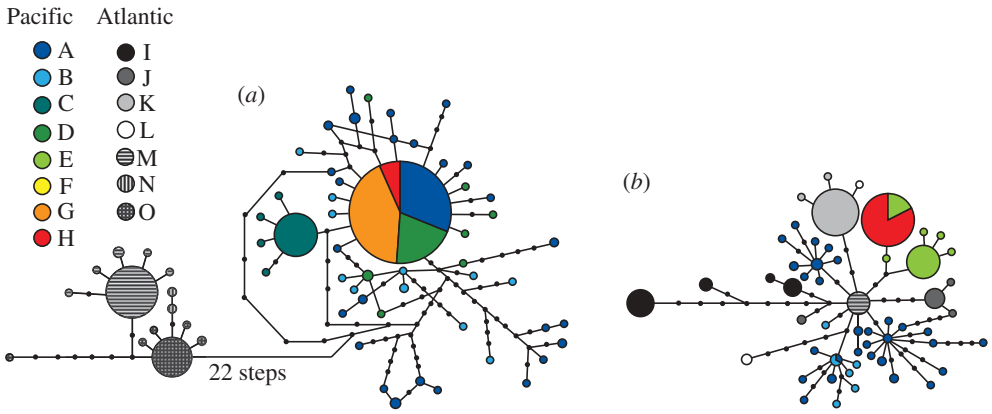

Figure 3. Mitochondrial haplotype networks of *Cerithideopsis californica* and *C. pliculosa*, subclades A1 and A2 (*a*), and subclade B1 (*b*) based on the CO1 gene. Circle sizes are proportional to the number of individuals observed for each haplotype. The small black circles represent unobserved single-nucleotide substitutions. The pie chart coloration/shading indicates the regions where haplotypes were collected (see electronic supplementary material, table S1).

Table 2. Analysis of molecular variance of *C. californica* in the Pacific (subclades A1 and B1) and *C. pliculosa* in the Atlantic (subclades A2 and B1).

ocean	clade	source of variation	d.f.	per cent of variation	Φ_{ST}
Pacific	subclade A1	among	11	24.82	0.25
		within	108	75.18	
		total	119		
	subclade B1	among	8	47.47	0.47
		within	69	52.53	
		total	77		
Atlantic	subclade A2	among	2	82.13	0.82
		within	47	17.87	
		total	47		
	subclade B1	among	7	90.41	0.90
		within	59	9.59	
		total	66		

improbable events become probable [5]. Our results indicate that, similar to the way terrestrial species can disperse over inhospitable water barriers to colonize land masses [47,48], marine species may disperse over land to colonize new oceans. While thousands of marine species were separated by the closure of the Central American Seaway, we show that marine species can occasionally breach this barrier despite the low probability of doing so. Several potential filters limit the probability of successful colonizations, similar to contemporaneous species invasions, which also involve a sequence of stages with sometimes independent probabilities of failure [49]. Natural dispersal events over land barriers by marine species are unlikely because individuals may not be picked up, survive the journey, survive in the novel environment, find mates or fail to establish purely owing to demographic stochasticity [50]. Thus, despite the fact that there are many migrating birds that regularly contact with snails, it seems improbable that snails would successfully disperse across Central America. Indeed, our genetic evidence supports this; although successful dispersal and establishment of these marine snails have occurred, it has rarely happened—being detected only two times within 3 Myr. We suspect that future molecular genetic data will reveal that other marine species, particularly common intertidal species, have probably also been dispersed across Central America by birds.

Reproductive incompatibility between geminate species pairs is not always complete as demonstrated by laboratory hybridization experiments between geminate species of gobioid fishes [51] and sea urchins [52,53]. We postulate that introgressive hybridization with 'native' individuals facilitated the retention and spread of the dispersed 'non-native' alleles that we detected. Despite being able to morphologically distinguish Pacific from Atlantic snails (O. Miura 2009, personal observation), there were no obvious morphological differences between individuals with the Pacific or the Atlantic mitochondrial haplotypes when they occurred in the same locality. Future analyses of highly variable nuclear markers (such as AFLP or microsatellites) could confirm the occurrence of introgressive hybridization between the Pacific and the Atlantic snails.

Charles Darwin first postulated that invertebrates, including marine snails, could be dispersed long distances by birds [7]. However, in contrast to terrestrial and freshwater invertebrates [8,54], there is little evidence for this for marine animals. Our genetic evidence coupled with evidence from field studies provide a conservative estimate that marine snails crossed Central America on two separate occasions, established their alleles, which subsequently spread along both coasts. This suggests that not only is such passive dispersal possible for marine organisms, but that it can occur across seemingly insurmountable barriers. Consistent with the emerging paradigm for island biogeography in which both vicariance processes and dispersal shape the distribution of terrestrial species [55], these marine dispersal events, while rare, could profoundly impact the ecology and evolution of marine species.

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