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Gene flow between Atlantic and Pacific Ocean basins in three lineages of deep-sea clams (Bivalvia: Vesicomyidae: Pliocardiinae) and subsequent limited gene flow within the Atlantic

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ABSTRACT

Pliocardiin (vesicomyid) clams rely on microbial symbionts for nutrition and are obligate inhabitants of deep-sea chemosynthetic ecosystems. Unlike many other invertebrate hosts of chemosynthetic microbes, pliocardiin clams are found in every ocean in a variety of reducing habitats, including hydrothermal vents, cold seeps, organic falls and deep-sea fans. The global distribution of pliocardiin clams suggests historical gene flow between ocean basins. We focus on 3 pliocardiin genera—*'Pliocardia'* I, *Calyptogena* and *Abyssogena*—each of which has a pair of sister clades in the Atlantic and Pacific. Our work tests the hypothesis that historical gene flow between the Atlantic and Pacific Oceans within these genera was interrupted by the closure of the Panamanian seaway and tests whether isolation between the ocean basins is the result of vicariance or past colonization. These questions are investigated in the context of fossil evidence, biogeography and phylogenetics. This study revealed a set of substitution rates consistent with other invertebrate studies ($\mu = 0.8\%$/My/lineage), and a set consistent with much lower rates often attributed to deep-sea organisms ($\mu = 0.3\%$/My/lineage). Among the Pacific/Atlantic sister pairs, *'Pliocardia'* I COI divergence per lineage is intermediate (2.5%), *Calyptogena* is the highest (6.1%) and *Abyssogena* the lowest (0.8%). The substitution rates suggest that *'Pliocardia' I* and *Calyptogena* have histories of at least 2.8 My in the Atlantic, with *Calyptogena* likely older. The slower rate, however, is inconsistent with both the maximum age of the family and several well studied fossils: leaving the faster rate preferred. With the faster rate, the *Abyssogena southwardae* clade diverged from its Pacific sister clade around 1 Mya, which likely post-dates the closure of the Isthmus of Panama and the opening of the Bering Strait. In light of this recent divergence, we test the previously proposed hypothesis that there is a high level of ongoing gene flow between Atlantic populations of *A. southwardae*. *A. southwardae* has colonized a broad geographic range of seep sites including the West Florida Escarpment, the Barbados Accretionary Prism, the Lobes of Congo, and the Mid-Atlantic Ridge north and south of the Romanche Transform Fault. Coalescent methods detect gene flow between Barbados and the Mid-Atlantic ridge; and between the West Florida Escarpment and the Lobes of Congo. All other comparisons failed to detect gene flow, contrary to prevailing interpretations of connectivity across the entire Atlantic Basin.

1. Introduction

Vesicomyid clams comprise two subfamilies: the relatively large Pliocardiinae which can reach more than 30 cm; and the Vesicomyinae that rarely grow beyond 1 cm (Krylova and Sahling, 2010). The Pliocardiinae rely on symbiotic chemosynthetic bacteria for nutrition while the Vesicomyinae lack the typical pliocardiin subfilamental tissues associated with symbiotic bacteria (Krylova and Sahling, 2010). Pliocardiin chemosymbiosis requires sulfide-rich reducing habitats such as whale falls (Smith et al., 1989), hydrothermal vents (Tunnicliffe, 1991), and cold seeps (Sibuet and Olu, 1998), including putative seeps at the terminal lobes of the Congo deep-sea fan (von Cosel and Olu, 2008). Some pliocardiin species, such as *Abyssogena southwardae*, are able to colonize multiple kinds of deep-sea sulfide-rich environments (Decker

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et al., 2012). In some places, populations of Pliocardiinae clams represent a large percentage of the biomass (Hashimoto et al., 1989; Olu et al., 1996). Dozens of pliocardiin species have colonized and maintained populations at patchy seeps and vents around the world. It is likely that pliocardiin larval dispersal capabilities play a key role in this widespread distribution and the long-term success of the group (Tyler and Young, 1999). Despite the importance of larval features in dispersal, very little is known about pliocardiin larvae other than observations of lecithotrophy (Duperron et al., 2013). Larval dispersal and recruitment of benthic organisms are also affected by abiotic factors in the water column (e.g. hydrography; Marsh et al., 2001) along with the ability to sense suitable substrata (e.g. sediment geochemistry; Shanks, 2009). Due to limited knowledge of dispersal capabilities in Pliocardiinae, historical dispersal is often studied by examining past gene flow (Teixeira et al., 2013; van der Heijden et al., 2012).

This study uses phylogenetic and population genetic methods to explore the histories of three pliocardiin lineages: '*Pliocardia*' I, *Calyptogena,* and *Abyssogena.* This and previous studies using the cytochrome c oxidase subunit I gene (COI) have identified Pacific/Atlantic sister taxa in each of three monophyletic pliocardiin lineages (Martin and Goffredi, 2012; Valdes et al., 2012). These sister taxa provide the opportunity to investigate historical gene flow between the Pacific and Atlantic with respect to two Pliocene events: the closure of the Isthmus of Panama and the opening of the Bering Strait. The objective of this investigation is to test hypotheses of when and how multiple lineages of Pliocardiinae arrived in the Atlantic. While the amount of COI divergence can help establish the relative timing of last contact between Pacific and Atlantic sister taxa, consideration of the fossil record allows estimates of absolute age.

Pliocardia, whose type species is the Jamaican fossil *P. bowdeniana,* has been difficult to assign to a monophyletic group of living species (Krylova and Sahling, 2006). Species tentatively assigned to *Pliocardia* are polyphyletic, falling into the monophyletic clades '*Pliocardia*' I and II (Valdes et al., 2012). Our study focused on the monophyletic '*Pliocardia*' I (Table 1). This group includes the East Pacific species *P. krylovata* located off of Costa Rica (Martin and Goffredi, 2012); *P. packardana* from the Monterey Canyon off California (Barry et al., 1997; Peek et al., 2000); and *P. stearnsii* from off the coast of California (Audzijonyte et al., 2012; Okutani et al., 2009). Nested within these East Pacific species is the Atlantic species *P. ponderosa* from the Gulf of Mexico (Martin and Goffredi, 2012). *P. ponderosa* and *P. krylovata* form a pair of sister species located in different ocean basins (Fig. 1). The discovery of this *'Pliocardia'* I Pacific/Atlantic sister pair nested within Pacific species strengthens the proposition by Amano and Kiel (2007) that *Pliocardia* has had a long history in the Pacific with the Jamaican fossil *P. bowdeniana* representing a relatively recent invasion of the Atlantic. Based on this finding, Amano and Kiel (2012) suggest that *P. krylovata* and *P. ponderosa* may have been separated by the rise of the Isthmus of Panama.

The second genus, *Calyptogena,* was re-examined with respect to shell morphology and COI phylogeny and found to be a clearly circumscribed *Calyptogena sensu stricto* (Decker et al., 2012; Krylova and Sahling, 2006). The species *C. valdiviae* is the most basal species in this group and is the sister species to all other *Calyptogena* (Decker et al., 2012). While most *Calyptogena* are found in the Pacific, *C. valdiviae* is from the Eastern Atlantic off of Africa (Table 1; Cosel and Salas, 2001; Thiele and Jaeckel, 1931; Valdes et al., 2012). The sister taxon to *Calyptogena s.s.* is *Elenaconcha guiness* which is also from the Eastern Atlantic off the coast of Africa (von Cosel and Olu, 2009). Our study focuses on the first Western Atlantic *Calyptogena s.s.,* namely *Calyptogena* n. sp. [Barbados], from a seep in the Barbados Accretionary Prism (Table 1; Fig. 1).

Table 1
Origin and number of previously published and newly received '*Pliocardia*' I and *Calyptogena* CO1 sequences used for this study.

Ridge System or Continental Margin	Habitat Type	Depth Distribution (m)
'Pliocardia' krylovata n = 2		
Costa Rica	Seep	740
'Pliocardia' packardana n =1		
C California Margin	Seep	635
'Pliocardia' ponderosa n =3		
Gulf of Mexico	Seep	737
'Pliocardia' stearnsii n =3		
Gulf of California	Seep	659–683
C California Margin	Seep	2200
Costa Rica Margin	Seep	2258–2263
C. fausta n =2		
Nankai Trough	Seep	1500–2200
C. gallardoi n =4		
Chile	Seep	750–1000
C. lepta n =4		
Gulf of California	Vent	2016–2020
C. makranensis n =1		
Indian Ocean	Seep	2215
C. mt-V *n*=1		
Costa Rica Margin	Seep	3096
C. pacifica n =27		
Oregon Margin	Seep	500–512
N California Margin	Seep	512
C California Margin	Seep	600–1000
Guaymas Basin	Seep	1560
C. sp. 3. n =1		
C California Margin	Seep	2895
C. starobogatovi-rectimargo n =29		
Juan de Fuca Ridge	Vent	1547–2437
C California Margin	Seep	1575
S California Margin	Seep	1825
Sea of Okhotsk	Seep	700
C. tuerkayi n =3		
SW Pacific Ocean	Seep	810
C. valdiviae n =1		
West African Coast	Seep	687
C. n. sp. [Barbados] *n*=18		
Barbados Accretionary Prism	Seep	1328

Fossil data is uncertain regarding the earliest appearance of *Calyptogena s.s.* A review of fossil *Calyptogena* suggests a late Miocene origin (≈ 7.2 Ma; Amano and Kiel, 2007), though the same authors later report a much older origin for *Calyptogena* in the Alaskan Oligocene *(C. katallaensis* ≈30 Ma; Kiel and Amano, 2010). Another *Calyptogena* fossil, *C. panamensis*, is found in the Burica beds of Pacific Costa Rica and Panama. Krylova and Sahling (2006) note that *C. panamensis* "very much resembles in proportion and gross outline" the extant Eastern Pacific *C. costaricana,* whose range coincides with *C. panamensis.* The Burica beds are reported to be at the boundary of the Miocene and Pliocene (5.3 Ma; Amano and Kiel, 2007; Krylova and Sahling, 2006), but this assessment did not consider the Coates et al. (1992) authoritative re-evaluation of Panamanian stratigraphy, which assigned the Burica beds to the Late Pliocene (2.6–3.5 Ma). From fossil data alone it is unclear when the Atlantic *Calyptogena* n. sp. [Barbados] was isolated from the Pacific.

The third monophyletic genus *Abyssogena* has been clearly circumscribed by Krylova et al. (2010). No fossil evidence is available for this genus. *Abyssogena* has five Pacific species known from previous COI studies and only one Atlantic member

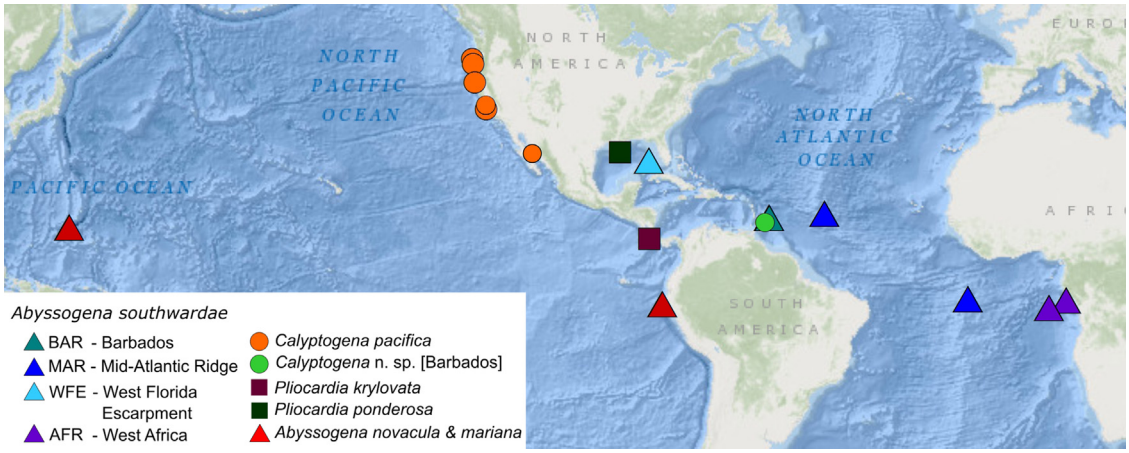

Fig. 1. Distribution of Pacific and Atlantic sister groups in '*Pliocardia*' I, *Calyptogena*, and *Abyssogena*.

Table 2
Origin and number of previously published and newly received *Abyssogena* CO1 sequences used for this study.

Ridge System or Continental Margin	Habitat Type	Depth Distribution (m)
A. kaikoi n =4		
Nankai Trough	Seep	3540–4800
A. mariana n =3		
Mariana Trench	Seep	5550–5861
A. novacula n =2		
Peru Margin	Seep	3500–5528
A. phaseoliformis n =10		
Kurile Trench	Seep	4700–6200
Japan Trench	Seep	5400–6400
Aleutian Trench	Seep	4550–6400
A. "Ryukyu Trench" *n*=2		
Ryukyu Trench	Seep	5900
A. southwardae n =139		
Barbados Accretionary Prism	Seep	4742–5000
West Florida Escarpment	Seep	3313–3288
N Mid-Atlantic Ridge	Vent	3038
S Mid-Atlantic Ridge	Vent	2995
West African Coast	Seep	3089–4946

(Table 2). *A. southwardae* is found across the Atlantic equatorial belt from the Gulf of Mexico to Western Africa, including the Mid-Atlantic Ridge (Fig. 1; Table 2; Krylova et al., 2010). Of all the taxa in the three focal lineages, only *A. southwardae* has multiple populations within an ocean basin and ample number of associated COI (Genbank sequences; Table 2). This enables both investigation of the Pacific/Atlantic divergence in this genus and application of coalescent methods to examine population dynamics (Audzijonyte et al., 2012; Lessios, 2008) We add COI data for specimens from two recently sampled populations of *A. southwardae* to the published *A. southwardae* COI sequences to reveal more of the overall genetic diversity of these populations (Zhang et al., 2010).

To examine historical gene flow in Pliocardiinae, we calibrated vesicomyid specific COI rates of substitution based on biogeography and fossil data. These rates were then used to consider the alternative hypotheses that the Atlantic member of each pliocardiin lineage considered here may have invaded from the Pacific following the opening of the Bering Strait ≈ 3.5 Mya. (Bigg et al., 2008; Vermeij, 1991; Wares and Cunningham, 2001), or through the Panamanian seaway, which was closed by the final emergence of the Isthmus of Panama ≈ 2.8 Mya (Lessios, 2008). COI substitution rates were subsequently used to examine gene flow between Atlantic populations of *Abyssogena southwardae* after the invasion of the Atlantic and test the hypothesis of high levels of gene flow between extant populations.

2. Materials and methods

2.1. Sample collection

Distribution of collection sites is shown in Fig. 1. General location, habitat type, and depth range for each species for which COI sequences were available is detailed for '*Pliocardia*' I and *Calyptogena* (Table 1) and for *Abyssogena southwardae* (Table 2). Latitude, longitude and depth of sample collections are presented in Supplementary Table 1. Phylogenetic analyses focused on the positioning of *Calyptogena* and *Abyssogena* species collected on two Seep-Connectivity (SeepC) expeditions in the Atlantic Ocean: R/V Atlantis cruise 21-02 to the Barbados Accretionary Prism in 2011 and R/V Atlantis cruise 26-15 to the Gulf of Mexico in 2014 (CL Van Dover, Chief Scientist). Clams were sampled using the ROV Jason and HOV Alvin and dissected foot or mantle tissues were preserved in 95% ethanol. From the Barbados Accretionary Prism tissues from 82 individuals of *Abyssogena southwardae* and 18 individuals of *Calyptogena* n. sp. [Barbados] were sequenced. From the Gulf of Mexico, tissues from 10 individuals of *A. southwardae* were collected and sequenced for this study. Additional sequences of published mitochondrial COI sequences were obtained from GenBank (Supplementary Table 1).

2.2. DNA sequencing

A portion of the Cytochrome c oxidase I (COI) gene was amplified by preforming polymerase chain reactions (PCR) using either the universal primers LCOI490 and HCO2198 (Folmer et al., 1994) or custom primers. These custom primers were designed from a *de novo* assembled transcriptome from *Abyssogena southwardae* using IDT PRIMERQUEST® (12 December, 2012). These primers did not overlap with the Folmer primers and the sequences were Forward 5′ CCCAAACCAGCAGGATCAA 3′ and Reverse 5′GGGTTTGATGGGAACTGCT 3′. Genomic DNA was extracted from ethanol-preserved specimens using the CTAB (CetylTrimethyl Ammonium Bromide) procedure (Doyle and Dickson, 1987).

PCR reactions were set up in a total volume of 25 μl containing 5 μl of 5xMyTaq Reaction Buffer (Bioline USA Inc.), 1 μl each of 10 μM forward and reverse primers, 1 μl of 10–100 ng/μl total DNA,

0.2 μl of myTaq DNA Polymerase (Bioline USA Inc.) and sterile water to the final volume. PCR thermal cycling conditions were as follows: 94 °C for 3 min, 35 cycles of 94 °C for 40 s, 48 °C for 40 s, 72 °C for 1 min with a final extension of 72 °C for 5 min.

PCR products were purified using 1 unit of Antarctic phosphatase (New England Biolabs) and 1 unit of exonuclease I (New England Biolabs) as per manufacturer's instructions and then sequenced in both directions using BigDye v1.1 Terminator reactions according to the manufacturer's protocol (Applied Biosystems: Foster, CA). Two sequencing reactions per individual (one for each primer) were performed by the Duke Genome Sequencing & Analysis Core Resource, using the ABI 3730xl DNA analyzer (Applied Biosystems). Depending on the laboratory, sequences were proofread in FinchTV 1.4.0 (Geospiza Inc.; Seattle, WA, USA) or Sequencher® version 5.4.1 (Gene Codes Corporation, Ann Arbor, MI USA http://www.genecodes.com).

2.3. Phylogenetic analysis and substitution rates

Pliocardiin COI sequences were obtained from three sources: clams from SeepC cruises, GenBank, and unpublished COI sequences collected by D. Jollivet over a period of years which were also useful to test identifications of GenBank collections. Sequences were trimmed to a shared 509 bp matrix. Automated model testing in Paup*4.0 (Swofford, 2003) was applied to two partitions; combined 1st/ 2nd codon positions and 3rd codon positions. Maximum likelihood trees were inferred from unique haplotypes by Garli 2.0 with 10 search replicates (Zwickl, 2006), allowing each partition its own model and substitution rate. 5000 bootstrap replicates were performed. To account for ancestral polymorphism (Edwards and Beerli, 2000), COI divergence was calculated beginning with the branch subtending the monophyletic group of interest. For example, in Fig. 4, the divergence between Clade 2 and Lineage 2 is calculated by summing the subtending branches A, B and C. Only subtending branches are shown in Supplementary Fig 1 when more than one individual was available. Substitution rates were calculated by plotting the percent substitutions per lineage (divergence; dependent variable) against the millions of years since divergence (independent variable). The slope of this correlation was calculated to determine the rate of substitution. To back calculate the millions of years since divergence for the three focal lineages, a linear regression (intercept of zero) was performed with percent substitutions per lineage as the independent variable. A time calibrated chronogram (Supplementary Fig 2) was produced with r8s 1.8 (Sanderson, 2003) and was based on maximum likelihood estimates of branch lengths from Garli (Zwickl, 2006). Cross validation was used to determine the optimal smoothing parameter of 10,000 (Sanderson, 2003). The chronogram was calculated by fixing the root of the tree at 58 Ma (Fig. 3b). All other nodes were not fixed.

2.4. Population genetic analysis

Four populations of Atlantic *Abyssogena southwardae* were studied in detail: West Florida Escarpment (WFE), Barbados Accretionary Prism (BAR), Mid-Atlantic Ridge (MAR) and Western African seeps (AFR). Haplotype diversity and nucleotide diversity θ, including an estimator based on pairwise distances ($\theta\pi$) (Tajima, 1983) and Ewen's estimator based infinite alleles model (θ_K) (Ewens, 1972), were calculated using Arlequin version 3.5 (Excoffier et al., 2005). Allelic richness (same as haplotype diversity in mtDNA), was further explored using a rarefaction model to remove the effect of different sample sizes (Kalinowski, 2005). Isolation and migration was estimated in each of 6 pairwise population comparisons using IMa (Hey and Nielsen, 2007). Under the HKY model, which is appropriate for mtDNA, mixing among 20 heated chains was monitored during trial runs, and the g1 and g2 parameters were adjusted in further runs to maximize mixing. Most runs worked well with g1 = 0.95 and g2 = 0.30. ESS was recorded as proxy for the number of independent parameter estimates. Multiple runs were carried out to narrow the priors. A median joining haplotype network (Bandelt et al., 1999) was also constructed for *Abyssogena southwardae* using PopART (Leigh and Bryant, 2015).

IMa post-analysis model testing was carried out on the final 100k of 500k genealogies sampled over 50 million iterative generations. For each pair of populations, likelihoods of 17 demographic models were compared by the AIC criterion (Akaike, 1976). These models estimate population parameters including population theta (q), migration rate m and time since population divergence.

3. Results

3.1. Identification of extant Pacific/Atlantic sister taxa

Our 2-model Garli ML analysis for our three monophyletic lineages adds new taxa and synthesizes insights from recent studies (Fig. 2; Audzijonyte et al., 2012; Decker et al., 2012; Martin and Goffredi, 2012; Valdes et al., 2012). A broader phylogenetic analysis was carried out for a larger selection of the Pliocardiinae, including all known representatives of the non-seep dwelling Vesicomyinae (Supplementary Fig 1; branch lengths here given for a single model for comparison to earlier studies). As with Valdes et al. (2012) and Martin and Goffredi (2012) *P. kryolovata* (Pacific) and *P. ponderosa* (Gulf of Mexico) form a trans-Isthmian geminate species pair, with the Californian *P. stearnsii/P. packardana* forming a clear sister group to the trans-Isthmian species pair (Martin and Goffredi, 2012). In *Calyptogena,* the newly-discovered *C.* n. sp. [Barbados] extends the range of *Calyptogena* to a single site in the Western Atlantic, and forms a trans-Isthmian geminate species pair with Pacific *C. pacifica* (Figs. 1 and 2). In both '*Pliocardia* I' and *Calyptogen*a, the restriction of present populations to the Caribbean Sea suggests cessation of gene flow between the Pacific and Atlantic may have preceded or coincided with the final closure of the Isthmus ≈ 2.8 Mya. (Lessios, 2008). In *Abyssogena,* two species from either side of the Pacific *(A. mariana* and *A. novacula)* form a sister group to the Atlantic *A. southwardae.* This Pacific/Atlantic clade is nested within two other Pacific clades of *Abyssogena (A. 'Ryukyu Trench'/A. kaikoi* clade and *A. phaseoliformis)*. The Pacific/Atlantic divergence in *Abyssogena* is much shallower than in '*Pliocardia*' I and *Calyptogena,* with *Calyptogena* the deepest of all (Fig. 2; see substitution rates below) and could suggest a pathway of Atlantic colonization besides the Isthmus of Panama in *Abyssogena*.

3.2. Pliocardiin rates of substitution

Rates of substitution in Pliocardiinae were calculated by applying fossil data and major geological events to the broader pliocardiin phylogeny which includes representatives from across both subfamilies (Supplementary Fig 1). Six lineages were dated using fossil evidence to estimate COI substitution rates (Table 3). The final closure of the Isthmus of Panama (at which time the Bering Strait was also open) was used to estimate substitution rates for our three extant pairs of Pacific/Atlantic sister taxa in '*Pliocardia*' I, *Calyptogena* and *Abyssogena.* The substitution rates fall into two classes when plotted (Fig. 3a). The first, tightest

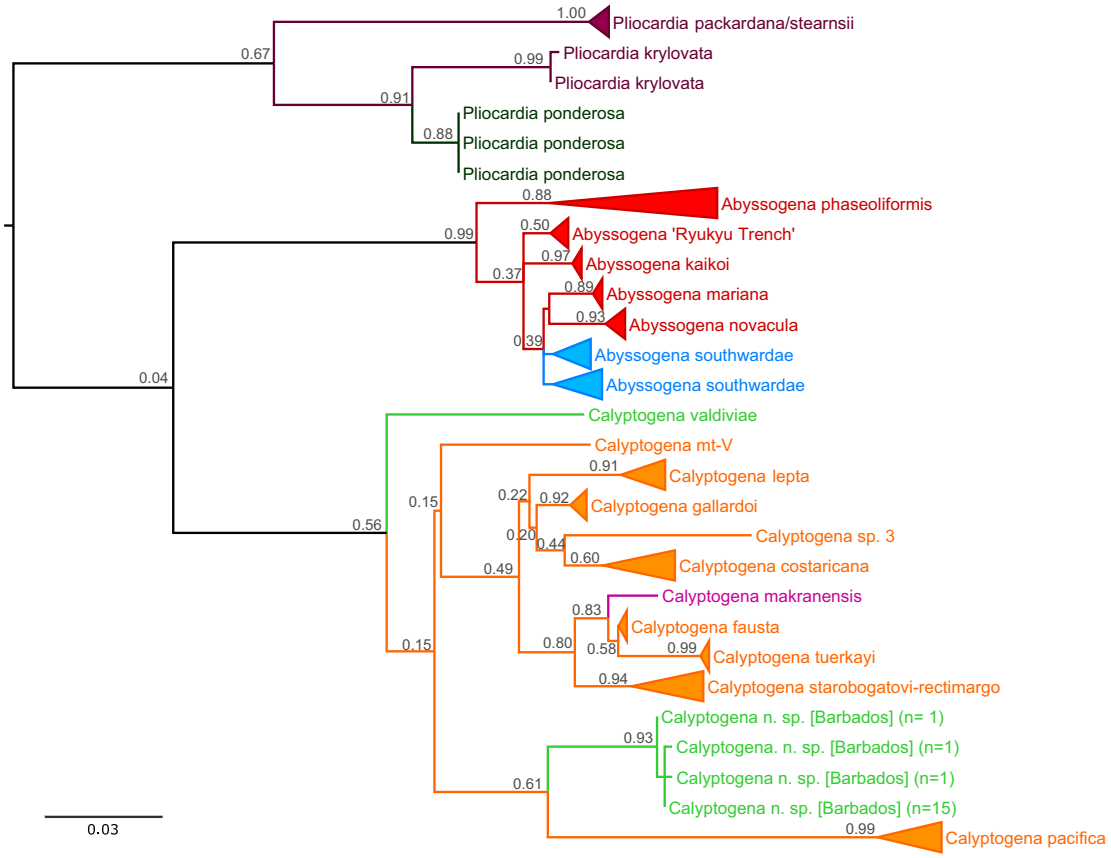

Fig. 2. COI Garli tree rooted with *Archivesica gigas,* with distinct models for 1st and 2nd codon positions versus 3rd codon positions. Results of 5000 bootstrap replicates are listed above the nodes. Colors represent species and ocean basin: Indian Ocean *Calyptogena* (Pink), Pacific *Calyptogena* (Orange), Atlantic *Calyptogena* (Green), Pacific *Pliocardia* (Purple), Atlantic *Pliocardia* (Dark-Green), Pacific *Abyssogena* (Red), Atlantic *Abyssogena* (Blue). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 3
Species comparisons used to calculate the rate of COI divergence and the basis for the age of the divergence. Data labels represent points in Fig. 3.

Data Label	Species Comparisons	Basis for age of divergence	COI Rate /Lineage/Ma.	Substitution Rate
Fossil Based Evidence				
A	***C. valdiviae* vs. all other *Calyptogena* s.s. lineages**	Amano and Kiel's (2007) estimate for late Miocene origin of *Calyptogena*	**5.4%/7.2 Ma**	**0.8%/Ma.**
B	***C. costaricana* vs. its closest relatives:*C. sp 3 and C. lepta***	Corrected age of fossil *C. panamensis*	**2.2%/3.1 Ma**	**0.7%/Ma.**
C	**Divergence between extant lineages of Pliocardiinae**	If *Isorropodon* is sister to extant Pliocardiinae, Amano and Kiel (2007) place the first *Isorropodon* at the Oligocene/Miocene boundary ≈23 Ma.	**18.4%/23 Ma**	**0.8%/Ma.**
D	***Archivesica gigas* vs. *Laubiericoncha myriamae***	*A. sakoi* ≈15 Ma.fossil	**7.0%/15 Ma**	**0.5%/Ma.**
E	**Divergence between extant lineages of Pliocardiinae**	Valdes et al. (2012) places the mid-Eocene pliocardiin fossils (≈45 Ma) at the common ancestor of extant lineages	**18.2%/45 Ma**	**0.4%/Ma.**
F	***C. valdiviae* vs. all other *Calyptogena* s.s. lineages**	Amano and Kiel's (2010) early Oligocene fossil *C. katallaensis*	**5.4%/30 Ma**	**0.2%/Ma.**
	Biogeography Based Evidence			
1	***P. krylovata* and *P. ponderosa***	Closure of Panamanian Isthmus ≈2.8 Ma.	**2.5%/2.8 Ma**	**0.9%/Ma.**
2	***C. pacifica* and *C. sp.* [Barbados]**	Closure of Panamanian Isthmus ≈2.8 Ma.	**6.1%/2.8 Ma**	**2.2%/Ma.**
3	***A.novacula* &*A. mariana* are the Pacific sister group to Atlantic *Abyssogena southwardae***	Closure of Panamanian Isthmus ≈2.8 Ma.	**0.8%/2.8Ma**	**0.3%/Ma.**

correlation has a faster μ of 0.8%/My/lineage (red data; Fig. 3a). The second, looser correlation consists of 4 dates consistent with a μ of 0.3%/My/lineage (blue data, Fig. 3a). These faster and slower rates were used to predict the age of divergence for the split between the Vesicomyinae and the seep-dwelling Pliocardiinae (Fig. 3b; 58 Ma vs. 130 Ma). The fast and slow substitution rate sets were then re-applied to the Pacific/Atlantic divergences in our lineages of interest (with percent divergence as the independent variable) (Table 4). In the chronogram (Supplementary Fig 2), when the divergence between the Vesicomyinae and Pliocardiinae is fixed at 58 Ma, the unconstrained nodes of interest (indicated with a red star) closely match the ages in Table 4 for the fast rate.

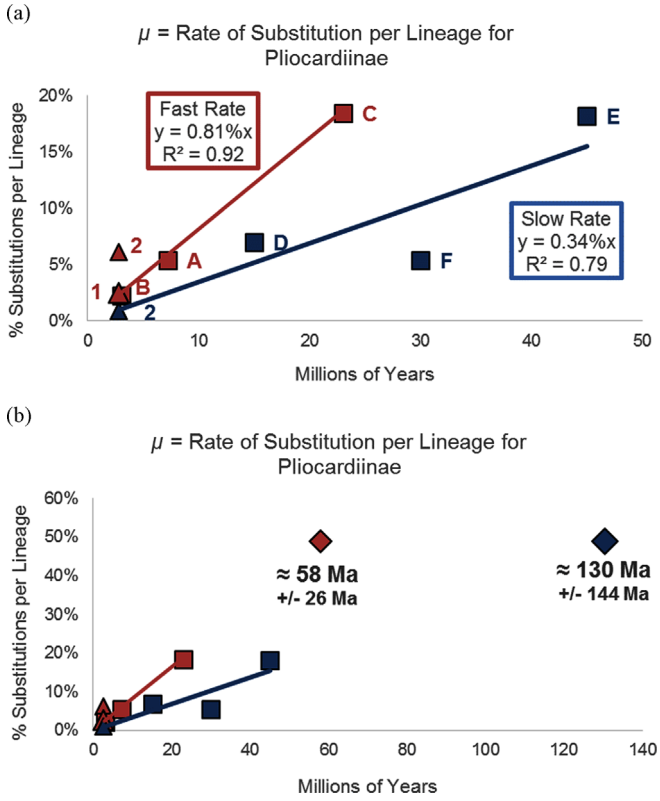

Fig. 3. Rate of substitution in Pliocardiinae based on fossil and biogeographic data. Squares represent fossil data, triangles represent biogeographic data and diamonds are extrapolated data points.

Table 4
Age of Pacific/Atlantic divergence based on slow and fast rate of substitution.

Predicted date (Mya)	Confidence Interval	Rate used
Divergence per lineage from *P. krylovata* to *P. ponderosa* = 2.5%		
2.96	4.9	Fast (0.8%/my)
6.52	28.9	Slow (0.3%/my)
Divergence per lineage from *C. pacifica* to C. n. sp. [Barbados] = 6.1%		
7.22	4.1	Fast (0.8%/my)
16.22	23.0	Slow (0.3%/my)
Divergence per lineage in *A. novacula*/*A. mariana* to *A. southwardae* = 0.8%		
0.95	5.5	Fast (0.8%/my)
2.13	32.9	Slow (0.3%/my)

Table 5
Population statistics calculated for Atlantic Abyssogena southwardae populations.

	N	Tajima's D	Haplotype Diversity	Allelic Richness[a]	θ_π	θ_K	IMa's Q***
AFR	34	−1.66*	0.82	4.6	2.3	8.4	39.7
WFE	13	1.30	0.51	2.8	0.5	0.4	1.5
BAR	81	−2.37**	0.64	3.7	0.9	7.9	53.9
MAR	11	−0.81	0.64	3.6	0.9	2.6	5.3

[a] rarefied to $N=8$.
* significant at 0.05.
** significant at 0.001.
*** averaged across all IMa comparisons including that population.

3.3. *Abyssogena southwardae genetic variation within populations*

A. southwardae has colonized four major areas across the Atlantic Ocean: the West Florida Escarpment (WFE), the Barbados Accretionary Prism (BAR), the Mid-Atlantic Ridge (MAR), and the Western African Coast (AFR; Fig. 1). Multiple populations have been sampled to some extent in all the regions except WFE, where only one population has been genetically sampled. Within-region sampling size is very uneven between populations: in the case of MAR only 8 individuals were collected across 2 widely separated populations (Logatchev and Clueless; ~4000 km apart). All standard measures of diversity agree that AFR has the most diversified population (Table 5). Comparison to allelic richness, which uses rarefaction to handle differences in population size, showed that both θ_K and IMa's Q were strongly influenced by sample size in BAR, whose true diversity was comparable to MAR for Haplotype Diversity, $\theta\pi$, and Allelic Richness, (Table 5). A haplotype network was also produced for additional visualization of the haplotype diversity (Supplementary Fig 3).

3.4. *Genetic divergence among populations of Abyssogena southwardae*

Genetic variation within *A. southwardae* was distributed among two clades of haplotypes – Lineages 1 and 2 (Fig. 4). Lineage 1 includes representatives of all four populations, including 3 sequences from AFR. Lineage 2 is confined to the Lobes of Congo population (AFR) (Fig. 4). A regional AMOVA revealed that 64% of this variation represents fixation among the four major regions (BAR, MAR, WFE, AFR; Table 6a), with only 37% of this variation accounted for within regions. The results are not changed when BAR and MAR were subdivided into intra-regional populations (Table 6b). Pairwise Fst values between regions were high and significantly different from zero (0.53 to 0.77 $p < 0.0001$; Table 6c). IMa estimates of time since divergence between major populations ranged from 130,000 to 1,140,000 years depending on the calibration used (Table 7; detailed IMa results are in Supplementary Table 2).

3.5. *Gene flow between populations in Abyssogena southwardae*

IMa was able to detect gene flow between only 2 (WFE:AFR and MAR:BAR) of the 6 pairs of populations (Table 8; Supplementary Table 2). Between AFR and WFE, IMa supports bidirectional gene flow with a $\Delta AIC < 2.0$ but rejects unidirectional gene flow from AFR to WFE. The phylogenetic evidence for gene flow between WFE and AFR comes from 3 AFR haplotypes that are nested in the WFE (clade 2 in Fig. 3). Similarly IMa supports bidirectional gene flow between MAR and BAR but rejects unidirectional gene flow from BAR to MAR. The phylogenetic evidence for gene flow between MAR and BAR comes from a shared haplotype between the two populations (Clade 1 in Fig. 3). For the remaining 4 pairs of populations, IMa Post-Analysis model testing rejected models with gene flow (> 2.0 ΔAIC), even between nearby populations such as WFE and BAR; Table 8). None of the population pairs with zero migration shared any alleles with each other.

4. Discussion

4.1. *Pacific/Atlantic vicariance in 'Pliocardia' I and Calyptogena*

The presence of both Pacific and Atlantic species in *'Pliocardia'* I, *Calyptogena,* and *Abyssogena* reveals the presence of past gene flow between these two ocean basins. In *'Pliocardia'* I and *Calyptogena* divergence between the Pacific and Atlantic species is most likely due to a vicariance event caused by the rising of the Isthmus of Panama at least 2.8 Mya. This is consistent with the known fossil record, biogeography and phylogeny of these genera.

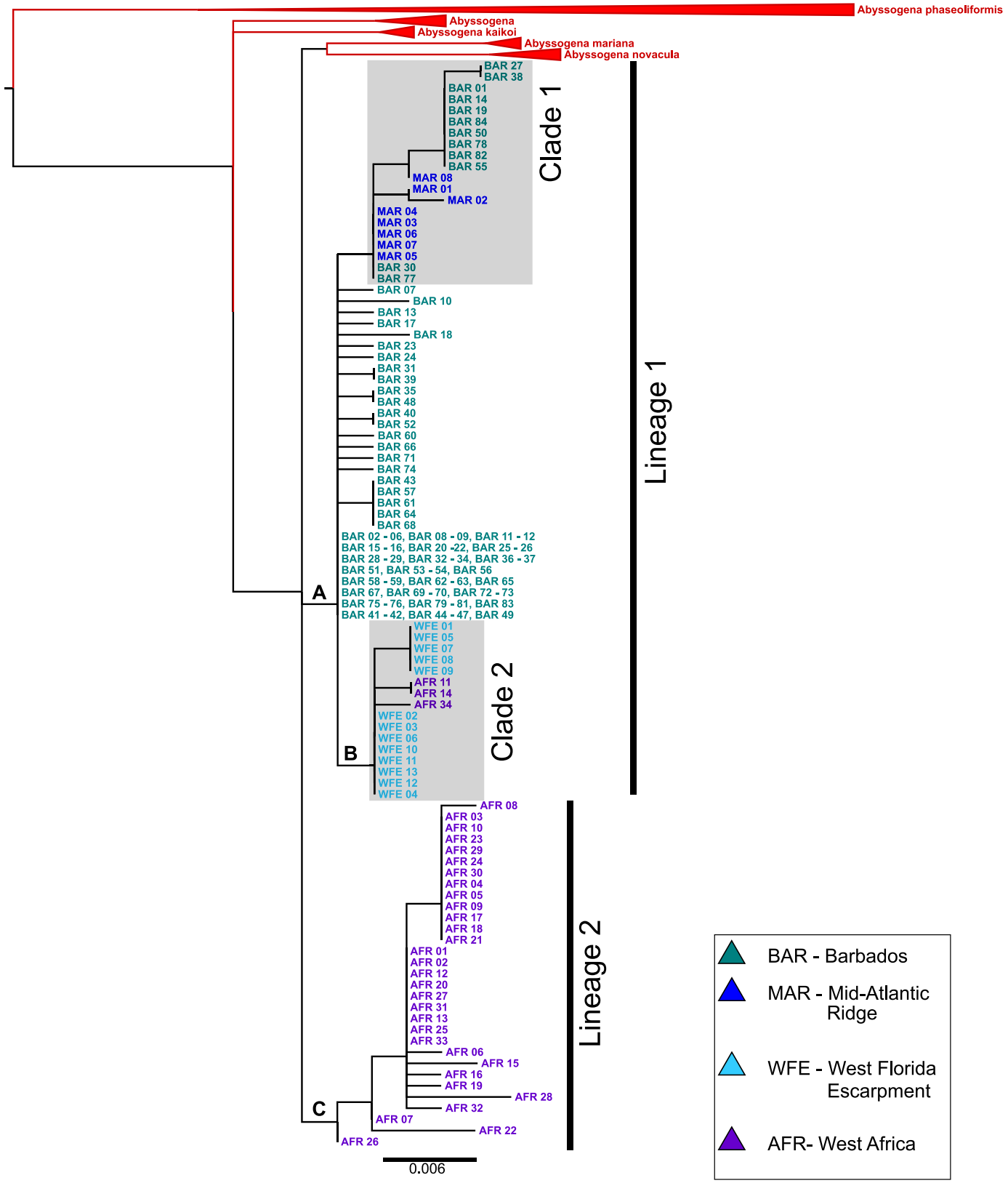

Fig. 4. Two-model Maximum likelihood Garli tree of *Abyssogena.* Pacific *Abyssogena* (Red) are collapsed as in Fig. 2. The Atlantic *Abyssogena* fall in two lineages denoted by vertical lines. Two notable clades within Lineage 1 are denoted with gray boxes. Branches labeled A, B and C are summed to calculate the divergence between Clade 2 and Lineage 2 while taking into account ancestral polymorphisms. (For interpretation of the references to color in this figure,the reader is referred to the web version of this article.)

Table 6
AMOVA results for (A) the main populations and the (B) same populations within Barbados (BAR) and the Mid-Atlantic Ridge (MAR) divided into sub-populations with the smaller sample representing between 25% (MAR) and 50% (BAR) of the total sample. Pairwise Fst's between the sample populations (C).

(A)

Source of variation	d.f.	Sum of Squares	Variance components	Percent of Variation	Fixation Indices	
Among Pops	3	82.7	1.04 Va	63.15	0.64	$p < 0.0001$
Fst Within Pops	132	80.3	0.61 Vb	36.85	0.06	$p \leq 0.076$ FSC

(B)

Source of variation	d.f.	Sum of squares	Variance components	Percent of variation	Fixation Indices	
Among Groups	3	82.6	1.04 Va	63.1	0.63	$p \leq 0.022$ FCT
Among Pops *within* Groups	2	1.2	0.001 Vb	0.03	0.001	$p \geq 0.10$ FSC
Within Pops	130	79.0	0.61 Vc	36.8	0.63	$p < 0.0001$ FST

(C)

Pairwise Fst's	AFR.	WFE	BAR
WFE	0.61**		
BAR	0.66**	0.56**	
MAR	0.64**	0.77**	0.53**
All significant	**< 0.0001**		

Table 7
Within-Abyssogena southwardae population ages of divergence. Percent divergence estimates were calculated in IMa. Divergence (in mya) was calculated with both slow and fast rate estimates (Table 3).

		AFR	WFE	BAR
WFE	% Divergence (IMa)	0.39%		
	Mya Divergence (fast 0.8%/my)	0.46[a]		
	Mya Divergence (slow 0.3%/my)	1.04[a]		
BAR	% Divergence (IMa)	0.30%	0.11%	
	Mya Divergence (fast 0.8%/my)	0.36	0.13	
	Mya Divergence (slow 0.3%/my)	0.80	0.29	
MAR	% Divergence (IMa)	0.30%	0.43%	0.24%
	Mya Divergence (fast 0.8%/my)	0.36	0.51	0.28[a]
	Mya Divergence (slow 0.3%/my)	0.80	1.14	0.64[a]

[a] Significant Gene Flow Between Populations from IMa.

Table 8
Bidirectional migration rates between Abyssogena southwardae populations from IMa, where Pop1 m = Pop2 m. Rates are presented forward in time.

		TO			
		AFR	**WFE**	**BAR**	**MAR**
FROM	**AFR**		0.10*	0[b]	0[b]
	WFE	0.10		0[b]	0[a]
	BAR	0[b]	0[b]		0.08**
	MAR	0[b]	0[†]	0.08	

For zeroes, IMa rejects
ANY detectable gene flow by
[a] > 2.5 ΔAIC or.
[b] 10 AIC.
* IMa rejects unidirectional gene flow AFR= > WFE > 6 ΔAIC.
** IMa rejects unidirectional gene flow BAR= > MAR > 8 ΔAIC.

A vicariance event resulting from the closure of the Isthmus of Panama in the pairs of sister taxa from '*Pliocardia*' I and *Calyptogena* is supported by their extant species distributions and fossil record. In both cases the sister species flank the Isthmus of Panama: the Pacific species in the Eastern Pacific and the Atlantic species in the Western Atlantic (Fig. 1.) This biogeographic pattern is typical of trans-Isthmian geminate species pairs (Lessios, 2008). To determine if vicariance across the Isthmus of Panama is consistent with the known fossil data, divergence dates were added to our phylogeny to calculate substitution rates (Table 3; Fig. 3). The fossil data fell into two sets: a set consistent with vicariance ~2.8 Mya in our sister taxa (blue line Fig. 3a); and a set with a much older date of divergence (red line Fig. 3a.) The slower estimated rate advocated by many authors (reviewed by: Vrijenhoek (2013)) is problematic. First, the hypothesis put forward that seep taxa have decreased the rate of substitution (Chevaldonne et al., 2002; Vrijenhoek, 2013) due to their chemosymbiosis (Vrijenhoek, 2010) or life history traits (McMullin et al., 2003) is not supported by the broader vesicomyid phylogeny: branch lengths of the sulfide-rich habitat dwelling subfamily Pliocardiinae are comparable to the non-sulfide-rich habitat dwelling Vesicomyinae (Supplementary Fig 1). Second, the slower substitution rate that fits the fossil *C. katallaensis* (Table 3) gives a divergence between *C. costaricana* and *C.* sp. 3/*C. lepta* that is twice as old as predicted by the *C. costaricana*-like fossil *C. panamensis* from the late Pliocene. Finally, the dating of the common ancestor of all extant lineages of Pliocardiinae at 45 Mya (Valdes et al., 2012) is problematic. This date is based on the oldest known vesicomyid, "*Archivesica*" *cf. tschudi*, which has several marked differences from the type species of *Archivesica* and cannot be assigned to any extant genera (Amano and Kiel, 2012). It is possible that this fossil represents a lineage that is not the common ancestor to the Pliocardiinae. The slower substitution rate also gives a divergence between the Vesicomyinae and Pliocardiinae in the Early Cretaceous (≈ 130 Ma). This is highly unlikely given that no vesicomyid fossils have been found older than 45 Ma. Furthermore, an Early Cretaceous divergence in the vesicomyids is questionable given the mid-Eocene to Miocene rise of modern vent and seep fauna, including vesicomyids in the late middle Eocene (Kiel, 2015). This shift in vent and seep fauna may be the result of a Paleocene-Eocene thermal maximum (Vrijenhoek, 2013) but has been more recently proposed to be the result of a strong increase in marine sulfate concentrations during the Eocene (Kiel, 2015). The faster substitution rate places the divergence of the Vesicomyinae and Pliocardiinae at 58 Ma. When this date is used to constrain the divergence between the subfamilies in a chronogram (Supplementary Fig 2) the unconstrained nodes are consistent with the faster rate and invasion of the '*Pliocardia*' I and *Calyptogena* prior to the final closure of the Isthmus of Panama.

Based on our analysis, we conclude that the fossil evidence is consistent with the faster rate of 0.8%/My. This rate gives a re-calculated Pacific/Atlantic divergence of 2.96 Mya in '*Pliocardia*' I and 7.22 Mya in *Calyptogena* (Table 4). These dates are consistent with gene flow through the Panamanian Seaway. The nesting of these Pacific/Atlantic sister taxon pairs within predominantly Pacific species (Fig. 1) suggests a longer history in the Pacific for these lineages and a recent invasion of the Atlantic. Longer Pacific history is also supported by fossil data. Pacific *Pliocardia* fossils have been discovered dating to the Oligocene and are absent from older seeps in the Atlantic (Amano and Kiel, 2012). No *Calyptogena* fossils have been discovered in the Atlantic and the fossil record for *Calyptogena pacifica* ranges to the late Miocene (Kanno et al.,

1989). This pattern of recent invasion is also seen in the *Abyssogena*.

4.2. Recent colonization of the Atlantic in Abyssogena

Based on the faster rate of substitution, the Pacific and Atlantic *Abyssogena* species diverged recently—less than 1 Mya. The biogeography of the *Abyssogena* sister taxa reveals very little about the possible route of gene flow between the ocean basins: *A. novacula* is found in the Eastern Pacific, *A. mariana* is found in the Western Pacific, and *A. southwardae* is found across the northern Atlantic Ocean. While the faster rate of substitution (divergence 0.95 Mya) excludes gene flow through the Panamanian Seaway, the slower rate of substitution (divergence 2.13 Mya) does not. Additionally both the Arctic and Antarctic are possible passages for *Abyssogena*. The Bering Strait would have opened prior to 1 Mya, connecting the Atlantic and Pacific (Bigg et al., 2008; Vermeij, 1991; Wares and Cunningham, 2001). The presence of extant Vesicomyidae in the Arctic (*Isoropodon* and *Laubiericoncha*) and Antarctic (*Vesicomya* and *Phreagena*) suggests that both routes are viable (Krylova and Sahling, 2010). It is difficult, however, to assess when stepping-stone seeps or vents were available to mediate Arctic or Antarctic migration. For example, fossilized seep communities containing *Isoropodon* and *Phreagena* clams were discovered on the Vestnesa Ridge in the high Arctic and likely lasted only 1000 years during the last deglaciation approximately 17,500 ya (Ambrose et al., 2015). While we cannot conclusively determine the route of gene flow between the Atlantic and the Pacific in *Abyssogena*, an invasion of the Atlantic less than 1 Mya is consistent with the population analysis of Atlantic *Abyssogena southwardae*.

4.3. Limited ongoing gene flow in the Atlantic Abyssogena southwardae

Within *Abyssogena southwardae* there are two nearly geographically distinct lineages. Lineage 1 contains all of the Barbados Accretionary Prism (BAR) samples and Lineage 2 contains almost all of the Western African (AFR) seep samples. This strongly suggests that lineage sorting is taking place and that Lineages 1 and 2 are close to reaching reciprocal monophyly in the mitochondrial gene (Fig. 4; Neigel and Avise, 1986). While both *Abyssogena southwardae* lineages appear to currently belong to a single interbreeding species, it is likely that at least two species will emerge in the Atlantic. This conclusion is consistent with a recent invasion of the Atlantic and establishment of multiple populations with a low level of ongoing migration. The polytomy at the base of these two lineages in the phylogeny is likely due to occasional ongoing gene flow between the lineages, which slows the process of lineage sorting (Neigel and Avise, 1986). This is supported by the absence or low level of detectable ongoing gene flow between populations of *Abyssogena southwardae* estimated in IMa. Furthermore, these estimates of ongoing gene flow are less consistent with *A. southwardae* invading the Atlantic over 2 Mya, as calculated with the slower rate, than with an invasion less than 1 Mya as calculated with the faster rate.

Populations of *A. southwardae* in the West Florida Escarpment and Western Africa seeps of the Lobes of Congo are the most geographically distant sites, yet IMa indicated some gene flow between these two populations. Although IMa was unable to reject bidirectional gene flow between WFE and AFR or unidirectional gene flow from WFE $=>$ AFR, it does reject unidirectional gene flow from AFR $=>$ WFE. Phylogenetic evidence is consistent with past gene flow from WFE $=>$ AFR: three of the AFR individuals in Lineage 1 fall into a clade formed by WFE haplotypes (see Clade 2, Fig. 4). Furthermore, the absence of a shared haplotype between AFR and WFE is consistent with this gene flow having happened tens of thousands of years in the past. Gene flow out of the Western African seeps in the Lobes of Congo is also not supported by the phylogenetic analysis (Fig. 4). The observation that Lineage 2 is entirely confined to AFR is consistent with an absence of directional gene flow from AFR to any sampled location. If successful migrants arrived in other regions from a source in AFR Lineage 2, those other populations would be represented in Lineage 2 – just as AFR is represented in Lineage 1. Since further sampling could change this conclusion, the evidence is weaker than the presence of the 3 AFR haplotypes nested within WFE in Clade 1, Lineage 1 (Fig. 4).

Although the Gulf of Mexico and Africa are the most geographically distant populations sampled in our study, gene flow between these regions has been suggested between the morphologically distinct *Escarpia laminata* and *Escarpia southwardae* (Cowart et al., 2013). There are two possibilities for this long range connectivity: the presence of undiscovered stepping stone environments (e.g. as yet undetected populations on the Mid-Atlantic Ridge) (Audzijonyte and Vrijenhoek, 2010) or larvae are carried by currents out of the Gulf of Mexico and into the deep equatorial jets of the Atlantic Ocean ultimately reaching the Eastern Atlantic (Ponte et al., 1990). While current knowledge of vesicomyid larvae gives little insight into their dispersal capabilities, models of other deep-sea invertebrate larvae have suggested that while connectivity between the Caribbean and the Gulf of Mexico is possible, neither location had the potential to send genes across the Atlantic Ocean (Young et al., 2012). This work also suggested that exchange across the Atlantic Ocean was likely only to occur in the east to west direction in the equatorial current system (Young et al., 2012). These models support the position that the AFR population is nearing reciprocal monophyly and that the gene flow seen in the phylogeny between AFR and WFE (Fig. 4) is due to incomplete lineage sorting and a recent common source population.

Shared haplotypes are a clear signature of gene flow and the only shared haplotype in our analysis of *Abyssogena southwardae* is found in Clade 1 between the Mid-Atlantic Ridge (MAR) and Barbados (BAR) populations. The MAR haplotype shared with BAR was found in Logatchev (Northern MAR) and one of 2 individuals collected at the Clueless vent (Southern MAR). The authors who collected these individuals concluded that there is no major hydrographic barrier between the Northern and Southern Atlantic Mid-Atlantic Ridge (van der Heijden et al., 2012). BAR and MAR also exhibit the lowest pairwise Fst of all the population comparisons supporting the conclusion that there is ongoing gene flow between the populations. The connectivity between BAR and MAR suggested by our analysis is consistent with models of larval transport in the region (Young et al., 2012).

The very low level of genetic diversity together with the undetectable or limited ongoing gene flow between populations of *Abyssogena southwardae* and the nearly reciprocally monophyletic lineages found on either side of the Atlantic suggests a recent step-by-step colonization of the Atlantic from a common source in the last million years. This is supported by recent divergences calculated between these populations (Table 7). The lowest percent divergence between populations (WFE and BAR) translates to a divergence of 130,000 ya (fast rate; 0.8%/my). The highest percent divergence (WFE and MAR) translates to a divergence of 510,000 ya (fast rate; 0.8%/my). This hypothesis is also supported in the Barbados and the West African seeps where the significantly negative Tajima's D values (Table 5) suggest population expansion after a bottleneck event such as colonization. It appears that *A. southwardae* is able to easily colonize new habitats and sustain these populations with limited or episodic subsequent gene flow between populations. This low level of gene flow is contrary to high connectivity and migration previously reported in this species (Teixeira et al., 2013). We conclude that the low genetic differentiation in *A. southwardae* is due to its recent origin in the

Atlantic Ocean and not a capability for frequent long-distance dispersal. Frequent long distance dispersal has also been suggested to underpin the global distribution of vesicomyids and the trans-oceanic distribution of several species (Decker et al., 2012; Krylova and Sahling, 2010; Teixeira et al., 2013). Our analysis suggests that the large lecithotrophic larvae of vesicomyids may use stepping stone habitats, instead of long distance dispersal, to cross vast geographic distances. The transient presence of *A. southwardae* on the Mid-Atlantic Ridge, with the Logatchev population disappearing over the course of a decade (Gebruk et al., 2000; Gebruk et al., 2010), is an example of a stepping stone that may have facilitated past gene flow that can no longer be sampled.

5. Conclusions

Based on a phylogeny of extant vesicomyids, fossil data and biogeography, we uncovered two very different rates of substation in COI. The faster of these two rates, 0.8%/my, is supported by the fossil data and the hypotheses that the sister taxon pairs in *'Pliocardia'* I and *Calyptogena* were the result of a vicariance caused by the closing of the Isthmus of Panama and that the low divergence in Atlantic populations of *Abyssogena southwardae* is due to a recent invasion followed by low ongoing gene flow. Unlike previous investigations (Teixeira et al., 2013), we do not find a high level of ongoing gene flow across the Atlantic Ocean. Additionally, we do not find significant support for the slowed substitution rates previously reported in deep-sea organisms (Vrijenhoek, 2013).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2016.08.013.

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