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Divergent sympatric lineages of the Atlantic and Indian Ocean crinoid *Tropiometra carinata*

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Abstract. The shallow water comatulid crinoid *Tropiometra carinata* is native to both the Atlantic and Indian Oceans, a distribution anomalous among shallow water crinoids and many other broadcast spawning species. Given this species' short pelagic larval duration, the findings of previous work that suggest that the Benguela upwelling is a significant barrier to gene flow in broadcast spawning species, and *T. carinata*'s unexpected geographic distribution, we predicted that the crinoids presently recognized as *T. carinata* consisted of a species complex. To test this prediction, we sequenced a portion of the mitochondrial cytochrome oxidase 1 gene from 30 individuals of *T. carinata* collected from Brazil, the Mozambique Channel, Madagascar, and Reunion Island. We found that nucleotide divergence ranged 0.02–3.10% among haplotypes. Moreover, while a Bayesian phylogenetic tree indicated that there were two substantially divergent genetic lineages, there was no evidence to support that *T. carinata* is comprised of a species complex due to isolation-by-distance. Surprisingly, both lineages were found in sympatry in both the Atlantic and Indian Oceans. Likewise, a 95% parsimony haplotype network revealed that identical haplotypes are found in both oceans, suggesting that a species complex may indeed exist, just not one caused by geographic isolation. We discuss possible explanations for this unexpected genetic structure, such as natural dispersal or human-mediated movement, and how the genetic structure found here is relevant to other marine organisms and to cryptic speciation.

Additional key words: pelagic larval duration, genetic divergence, Benguela Current

The extent of the dispersal capabilities of many marine broadcast spawning species and the biogeographic processes underlying their current distributions have historically been hard to assess due to the inherent difficulties of studying marine taxa. Direct observations of larval dispersal that utilize chemical labels are difficult due to the small sizes of larvae, and low recapture rates (Levin 2006; Cowen & Sponaugle 2009). Therefore, gene flow estimates among many populations have been based on combinations of larval presence, known or estimated pelagic larval duration (PLD), and ocean current patterns (Scheltema 1971; Jablonski 1986). More recently, studies using analyses of genetic data have helped in estimation of gene flow among regions, elucidating the patterns of and processes behind species distributions (Perrin et al. 2004; Hunter & Halanych 2008; Ayre et al. 2009; Zulliger et al. 2009). Some molecular genetic studies have provided added support to previous hypotheses concerning barriers to gene flow between populations, such as isolation-by-distance, prominent geographic features, or strong currents (Waters & Roy 2003; Baus et al. 2005; Puebla et al. 2009). Other genetic studies have helped to identify barriers to gene flow when no obvious barrier was suspected (Ayers & Waters 2005; Keever et al. 2009). In the past, it was assumed that "cosmopolitan species" had the capabilities to disperse over great expanses, but studies using genetic data have shown significant genetic divergence between populations due to isolation-by-distance, and even suggest cryptic speciation (Knowlton 1993; Klautau et al. 1999; Howell et al. 2004; Colgan et al. 2005; Zulliger & Lessios 2010).

Most species in the echinoderm class Crinoidea are dioecious. Their reproductive strategies vary, with some species broadcast spawning both eggs

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and sperm, while others brood eggs or larvae internally or externally (Messing 1984; Holland 1991). However, all broadcast spawning crinoids have nonfeeding, free-swimming larvae that settle and metamorphose into a stalked sessile form (pentacrinoid), with some (comatulids, commonly referred to as "feather stars") breaking away to become free-moving adults (Anderson 2001). While adult comatulids are capable of some swimming, movement is usually used only for avoiding predation, finding shelter from strong currents or waves, or finding better feeding perches (Meyer & Macurda 1977). Some studies have suggested that adult migration via rafting is a possibility (Marr 1963; Waters 2007), but a recent literature review of rafting marine organisms only found one instance of rafting by a crinoid (Thiel & Gutow 2005). Furthermore, this review suggested that dioecious or broadcast spawning species, in general, are not well-suited to colonize new areas via rafting. Due to these limitations, a crinoid species' pelagic larval stage is often considered its only means of natural, long distance dispersal (Emlet 1995). Species with relatively long PLDs, upwards of 20 d, are expected to disperse farther than those with short PLDs (Marshall & Keough 2003). A genetic study by Wilson et al. (2007) suggested that populations of the "circumpolar" crinoid, *Promachocrinus kerguelensis* Carpenter 1888, on the western side of the Antarctic Peninsula are composed of five separate cryptic species, leading them to doubt previous estimates of a 3 month PLD in this species (McClintock & Pearse 1987). Perhaps the most interesting result of this study was that most of the putative species were found in sympatry throughout their sampling range, which Wilson et al. (2007) hypothesized may be due to the isolation of *Promachocrinus* spp. populations over the last 4 million years, resulting in allopatric speciation, and subsequent recolonization events after the last glacial maxima ~20 kya.

In direct contrast with *P. kerguelensis*, the shallow-water tropical crinoid *Tropiometra carinata* Lamarck 1816 is known to have a wide geographic distribution (Fig. 1; Messing 2010a). It is a broadcast spawning species with a relatively short PLD of 2–9 d (Mortensen 1920). The presence of *T. carinata* along both the eastern coast of South America in the Atlantic Ocean and on the eastern coast of Africa in the Indian Ocean is unusual; other tropical comatulid crinoids have substantially more restricted geographic ranges (C. Messing, pers. comm.). Also, like many echinoderms, *T. carinata* includes several color morphs. In Brazil, some individuals are solid black, while others are black with white stripes (M.D. Correia, pers. obs.). Clark (1917) noted that individuals in the Caribbean have colors ranging from dark purple to reddish-brown with varying degrees of striping. In the Indian Ocean, however, individuals are often bright orange and yellow with dark stripes (Lanterbecq et al. 2003).

In this study, we sought to examine genetic differentiation within and among geographically disjunct populations of *T. carinata* using traditional phylogenetic techniques. First, we predicted that the two color morphs found in Brazil would not show any significant genetic differentiation. While some studies have suggested the existence of different lineages or species for marine invertebrates based on color polymorphism, many others have noted that color variation among species is common and could be a result of diet, exposure to light, behavioral patterns, or age (Calderón et al. 2010). Second, we hypothesized that the two geographically disjunct populations of *T. carinata* found in the Atlantic and Indian Oceans would show significant genetic divergence. Previous studies of broadcast spawning species have shown that the tip of the African continent, where the cold Benguela Current flows, is a significant barrier to gene flow for species with pelagic larvae (Lessios et al. 1999, 2003). Moreover, in a study of pantropical sea urchins in the genus *Diadema*, Lessios et al. (2001) found that the Atlantic Ocean's *Diadema antillarum* Philippi 1845 differed from the Indian Ocean's *D. paucispinum* Agassiz 1863 and *D. savignyi* Audouin 1829, respectively, by 3.23% and 2.96% mean percent base pair substitutions at a mtDNA locus that included the cytochrome oxidase 1 (COI) gene. Considering the relatively short PLD of *T. carinata*, its tropical range, and the findings of previous studies, we predicted that we would find lineages with genetic divergence similar to that of other echinoderm species found in the same region (e.g., *Diadema* spp.), which would indicate the need for further research to examine whether the "populations" of *T. carinata* actually comprise a species complex. Therefore, we compared genetic differentiation between the Atlantic and Indian Ocean populations of *T. carinata* with other pantropical, congeneric species. We conclude by discussing the results of our findings and how natural or human-mediated dispersal might be responsible for the observed divergent lineages of *T. carinata*.

Methods

Sampling

Twenty specimens of *T. carinata* were collected haphazardly by snorkeling divers from the Saco da

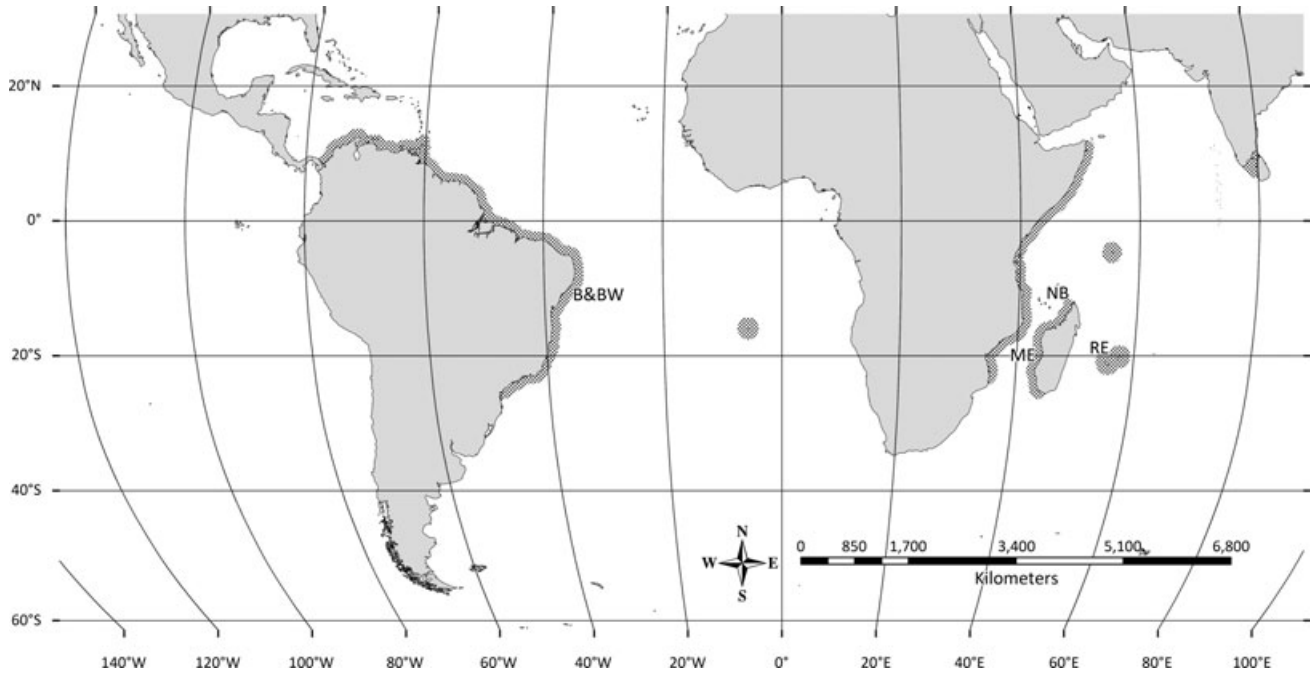

Fig. 1. The known range of *Tropiometra carinata* (gray shading) and approximate sampling locations for the specimens examined in this study. B and BW, Alagoas, Brazil; ME, Iles Esparses, Mozambique Channel; NB, Nosy-Bè, Madagascar; RE, Reunion Island, Indian Ocean.

Pedra sandstone reef in Alagoas State, Brazil in December 2009 (Fig. 1). These sandstone reefs are known to have a wide diversity of intertidal and subtidal species as well as seasonally varying salinity levels (Correia 2011). The specimens of *T. carinata* collected in Brazil included members of the two different color morphs, solid black (B) and black with white stripes (BW). Our collection consisted of ten of each color morph to determine if color variation correlated with genetic lineage. One arm was removed from each individual and immediately preserved in Drierite® desiccant (WA Hammond Drierite Co., Xenia, OH, USA). Cytochrome oxidase 1 sequences from 11 additional individuals of *T. carinata* from the Mozambique Channel, Madagascar, and Reunion Island in the Indian Ocean (Fig. 1), and a COI gene sequence from an individual of *T. macrodiscus* Hara 1895 (Messing 2010b) collected from Japan were made available to us from the Florida Museum of Natural History, Gainesville, Florida (Table 1). The color morphs of the Indian Ocean specimens were not cataloged with the museum specimens.

DNA extraction/PCR

Genomic DNA was extracted from tissue obtained from the proximal portion of each arm using a Qiagen DNeasy® blood and tissue kit (Qiagen, Valencia, CA, USA). Using the primer pair COIceF (5′-ACTGCCCACGCCTAGTAATGATA TTTTTTATGGTNATGCC -3′) and COIceR (5′-TCGTGTGTCTACGTCCATTCCTACTGTRAAC ATRTG-3′) described by Hoareau & Boissin (2010), a portion of the COI gene was amplified in 20 μL polymerase chain reactions containing 1.5 μL of 2.5 mM $MgCl_2$, 1.6 μL of 10.0 mM dNTPs, 2.0 μL 10× PCR buffer, 1.0 μL of each 10.0 μM primer solution, 0.2 μL of 1:10 Taq, and 1.0 μL of 50–100 ng DNA template. The PCR thermocycler program began with an initial denaturing step of 4 min at 94°C, and continued with 35 cycles of denaturing at 94°C for 30 s, annealing at 49°C for 30 s, and extension at 75°C for 45 s. The program ended with a final extension at 75°C for 5 min. Amplification of the PCR products was verified by electrophoresis on a 2% agarose gel containing ethidium bromide. The products were purified using Exo-SAP IT (USB Corp., Cleveland, OH, USA) and sequenced in forward and reverse directions using an Applied Biosystems 3730XL DNA Analyzer (University of Arizona, Tucson, AZ, USA).

Data analyses

Chromatogram contigs for the Brazilian population were inspected and assembled by eye, and nucleotide sequences from all regions were aligned

Table 1. Collection site and Genbank accession numbers for the specimens of *Tropiometra carinata*, *T. macrodiscus*, and *Florometra serratissima* examined in this study.

Sample ID	FMNH ID	Genbank accession #	Collection location
B1–B10	n/a	JX236067 – JX236075	Alagoas, Brazil
BW1–BW10	n/a	JX236076 – JX236085	Alagoas, Brazil
ME1	9220	JX236086	Iles Esparses, Mozambique Channel
ME2	9250	JX236087	Iles Esparses, Mozambique Channel
ME3	9255	JX236088	Iles Esparses, Mozambique Channel
NB1	7355	JX236089	Nosy-Bè, Madagascar
NB2	7362	JX236090	Nosy-Bè, Madagascar
NB3	7548	JX236091	Nosy-Bè, Madagascar
NB4	7549	JX236092	Nosy-Bè, Madagascar
NB5	7560	JX236093	Nosy-Bè, Madagascar
NB6	7562	JX236094	Nosy-Bè, Madagascar
RE1	REU-0064	JX236095	Reunion Island, Indian Ocean
RE2	6577	JX236096	Reunion Island, Indian Ocean
T. macrodiscus	3873	JX236097	Ryukyu Islands, East China Sea
Florometra serratissima	n/a	AF049132	British Columbia, Canada

using Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, MI, USA). All other sequences were visually checked and edited using GENEIOUS® software (http://www.geneious.com). The aligned COI sequences from each region varied slightly in length; sequences were trimmed to 557 bp to allow for congruent sequences during analyses. For phylogeny reconstruction, we first determined the model of evolution that was the best fit for the data according to AIC calculations implemented by MrModeltest 2.3 (Nylander 2004). The results indicated the best fit model of evolution for our data was the general time-reversible model combined with the model of gamma distribution (GTR + G). Despite the protein-coding nature of COI, we used unpartitioned data due to a scarcity of informative characters. We used MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) to estimate a Bayesian Metropolis-coupled Markov chain Monte Carlo phylogeny using unique haplotypes. Each run was comprised of four chains and sampled every 100 generations for 1×10^7 generations. Tracer v1.5 (Rambaut & Drummond 2007) was used to check for stationarity. The first 2.5×10^4 generations were conservatively discarded as burnin. To determine whether color morph or geographic regions were correlated with lineage diversification, we visually examined how these characteristics mapped onto the phylogeny. The outgroups for the phylogenetic tree were comprised of the sister taxa *T. macrodiscus* (Table 1), and another comatulid, *Florometra serratissima* Clark 1907. Additionally, to characterize intraspecific relationship among haplotypes, we created a 95% statistical parsimony haplotype network as implemented in TCS 1.21 (Clement et al. 2000). We calculated within-group means for each region with MEGA 4.0.2 (Kumar et al. 2008). Finally, to compare genetic divergence between sampling locations and unique haplotypes, we calculated mean percent-nucleotide distances using the Kimura two-parameter (K_2) method with MEGA 4.0.2 (Kimura 1980; Kumar et al. 2008).

Results

We obtained high-quality sequence data from 19 of the 20 specimens of *Tropiometra carinata* from Brazil (Genbank accession JX236067–JX236085). The sequence analysis using Sequencher v.4.1.4 (Gene Codes Corp.) indicated that the sequences contained no stop codons, which is expected of the mitochondrial COI gene, and indicated that the sequences are the actual COI gene and not a pseudogene.

The Bayesian phylogenetic tree exhibited two lineages within *T. carinata* (Fig. 2). Lineage 1 contained individuals from Brazil, Mozambique Channel, and Madagascar, whereas Lineage 2 contained only three individuals (one from Brazil, and two from Reunion Island). Additionally, Lineage 2 splits further, with the Brazilian sample (BW7) diverging prior to the two Reunion Island individuals.

The TCS1.21 (Clement et al. 2000) analysis could not link all haplotypes into a single network with 95% confidence, having a 95% parsimony probability connection limit up to 10 steps. Rather, the two lineages described above were reconstructed as two independent networks (Fig. 3). Only one haplotype,

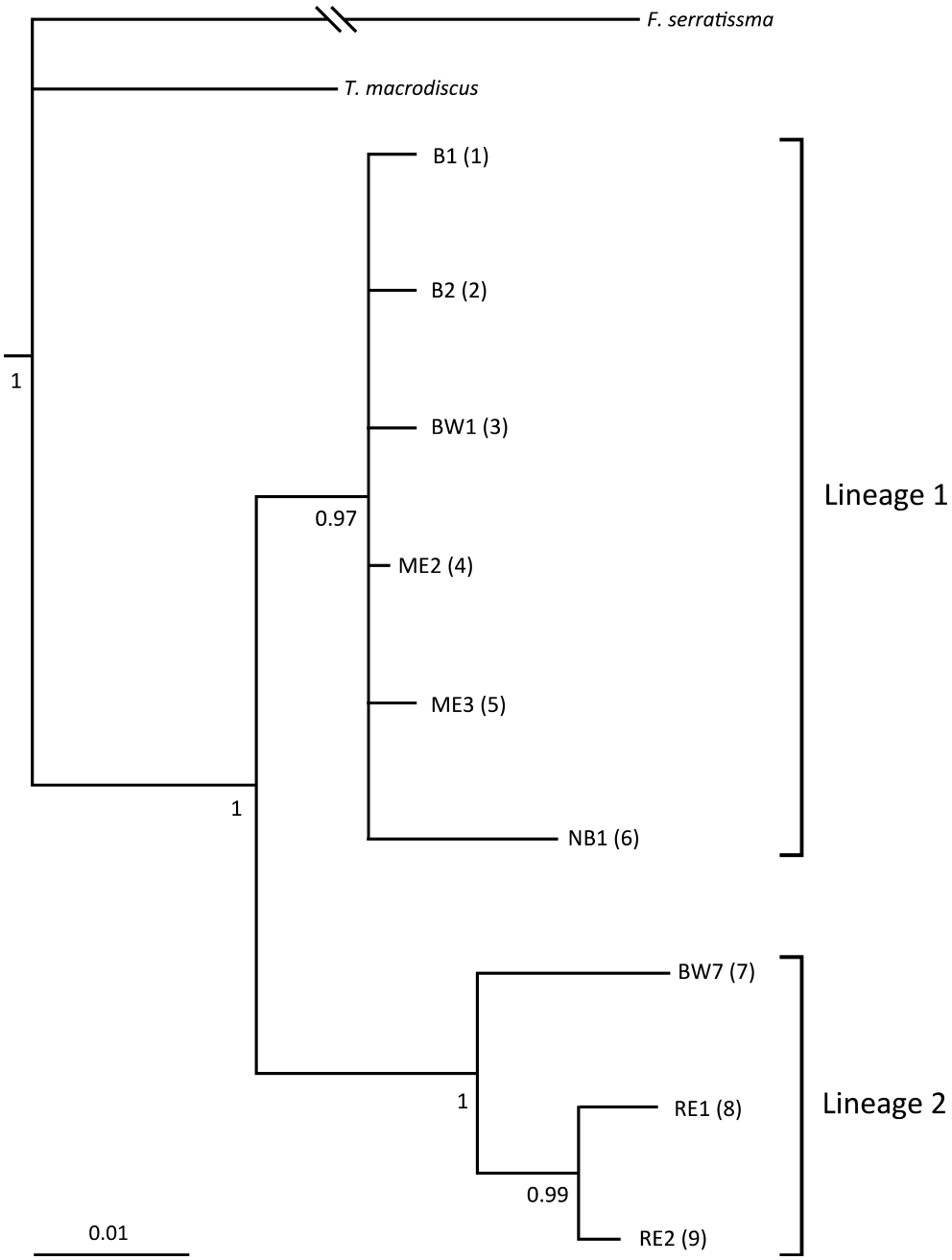

Fig. 2. Bayesian phylogram showing relationships of the specimens of *Tropiometra carinata* examined in this study. Numbers at each node indicate posterior probabilities. The numbers in parentheses indicate assigned numbers given to unique haplotypes, which correspond to the haplotype network shown in Figure 3. The scale bar indicates the number of nucleotide substitutions per site.

the putative ancestral haplotype, was found in more than one sampling location. The Brazilian outlier (BW7) is distantly linked with the Reunion Island individuals in the second network. Moreover, there was no evidence of differentiation among color morphs, with both Brazilian color morphs sharing identical haplotypes.

Within-group means for percent nucleotide divergence were low (unweighted average of 0.3% sequence divergence) within each region, with BW7 excluded from the Brazilian population and treated as its own group (Table 2). Reunion Island exhibited the largest percent nucleotide distance. In contrast, between-group means were as high as 3.1%

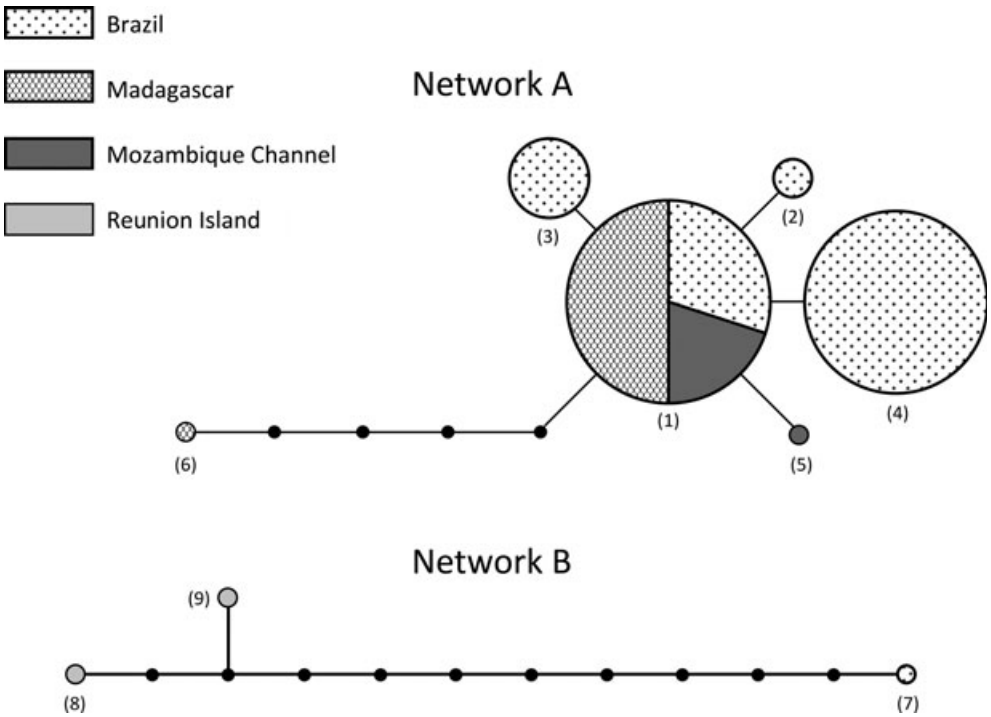

Fig. 3. Haplotype network of *Tropiometra carinata* populations. The small black circles indicate the number of mutations (missing haplotypes) between each haplotype. The proportional sizing of the haplotype circles indicates the number of individuals with that haplotype, and the numbers in parentheses indicate assigned numbers given to unique haplotypes, which correspond to the Bayesian phylogram shown in Figure 2.

sequence divergence. Additionally, the percent nucleotide divergence between unique haplotypes ranged 0.18–4.07% (Table 3). While the sample sizes from Brazil were sufficiently large, the sequences from the Indian Ocean came exclusively from museum specimens and were the only samples of *T. carinata* available for analysis. However, the pairwise genetic divergence between the unique haplotypes is revealing, and our phylogenetic analyses are strongly supported despite the small sample size.

Discussion

Comparison of genetic variation within and among populations of *Tropiometra carinata* from the Atlantic and Indian Oceans revealed that *T. carinata* is comprised of two genetically divergent lineages (Figs. 2, 3), yet there is no evidence to suggest that these two lineages evolved due to isolation-by-distance. The haplotype network and phylogenetic tree revealed that one haplotype found in

Table 2. Kimura-2-parameter percent nucleotide divergences at the cytochrome oxidase I locus between populations of *Tropiometra carinata* and its congener *T. macrodiscus*. Among-population/-species comparisons are below the diagonal, and within-group means are in bold. Individual BW7 was not included in the Brazil population when calculating percent nucleotide distances.

	Brazil	BW7	Mozambique Channel	Madagascar	Reunion Island	*T. macrodiscus*
Brazil	**0.20**					
BW7	3.10	**n/a**				
Mozambique Channel	0.20	3.00	**0.10**			
Madagascar	0.30	3.10	0.20	**0.40**		
Reunion Island	2.60	2.00	2.50	2.70	**0.50**	
T. macrodiscus	3.80	4.80	3.80	3.90	4.90	**n/a**

Table 3. Kimura-2-parameter percent nucleotide divergences at the COI locus between unique haplotypes of *Tropiometra carinata* and its congener *T. macrodiscus*.

	B2	BW1	ME2	B1	ME3	NB1	BW7	RE1	RE2
BW1	0.36								
ME2	0.18	0.18							
B1	0.36	0.36	0.18						
ME3	0.36	0.36	0.18	0.36					
NB1	1.27	1.27	1.09	1.27	1.27				
BW7	3.13	3.13	2.94	3.13	3.13	4.07			
RE1	2.94	2.94	2.75	2.94	2.94	3.89	2.01		
RE2	2.38	2.38	2.19	2.38	2.38	3.31	1.83	0.54	
T. macrodiscus	3.88	3.38	3.69	3.88	3.88	4.83	4.83	5.03	4.83

Lineage 1 (Haplotype 1; Figs. 2, 3) occurred in the Atlantic and Indian Oceans and was the putative ancestral haplotype. Lineage 2 was comprised of only a few samples, and additional sampling may shed more light on patterns of diversification. While the Bayesian phylogeny suggests a relatively deep split between Lineage 1 and Lineage 2 (Fig. 2), there is a more recent split between the Reunion Island haplotypes and the Brazilian BW7 haplotype within Lineage 2. These patterns of diversification were surprising considering the findings of previous genetic studies looking at broadcast spawning species within the same region. For example, studies of the pantropic sea urchin genera *Eucidaris*, *Tripneustes*, and *Diadema* have shown substantial divergence between the COI haplotypes of Atlantic and Indian Ocean species (Lessios et al. 1999, 2001, 2003). Likewise, similar results have been found in genetic studies of other taxa with planktonic larva such as *Echinolittorina* (periwinkles), *Palinurus* (spiny lobsters), and *Maja* (spider crabs) (Williams & Reid 2004; Palero et al. 2009; Sotelo et al. 2009).

Therefore, finding no evidence of speciation due to isolation-by-distance raises the following questions: What mechanisms enabled the split of the two genetic lineages? And, are the lineages so distinct that they comprise different (cryptic) species? In considering whether the genetic pattern can be explained by allopatric speciation, it is important to assess the biogeographic history of this species. According to Wilson et al. (2007), a generalized molecular clock for the echinoderm COI gene was estimated to be 3.1–3.5% sequence divergence per million years (MY). This would crudely date the split between the Reunion Island/BW7 lineage and the Brazilian/Mozambique Channel/Madagascar lineage at approximately 1 MY since divergence. Despite our inability to assess which lineage evolved in which region, the strong Benguela upwelling, which became a permanent feature during the late Pliocene, is hypothesized to have been the cause of allopatric speciation for many broadcast spawning species (Lessios et al. 1999, 2003; McCartney et al. 2000). The cold Benguela upwelling was estimated to have weakened around 1.6 mya, and has experienced cyclical fluctuations in strength since that time (Lessios et al. 2001).

Subsequent to lineage diversification, individuals would have had to disperse between separated regions. It is possible that *T. carinata* has dispersed naturally, and that its larvae are hardy enough to withstand transport past the upwelling during the times when the upwelling is weak. Still, *T. carinata's* short PLD is not long enough to account for its distribution across the Atlantic (Marshall & Keough 2003). Unlike many other Atlantic echinoderms, *T. carinata* is not known to be present along the west coast of Africa, adding support to the estimate of PLD for *T. carinata*. However, Mortensen (1920) estimated the PLD for larvae of *T. carinata* in glass jars, which could have produced erroneous times relative to more natural conditions. Indeed, the Smithsonian National Museum of Natural History collection contains individuals of *T. carinata* (ID numbers 17471, E20793) collected from Saint Helena in the South Atlantic Ocean, providing evidence contrary to Mortensen's observation. It is possible that the PLD of *T. carinata* is substantially longer than 9 d, but that the shallow waters off the west coast of Africa are not an adequate habitat for this species. Conversely, if *T. carinata*'s PLD is indeed ~9 d, its dispersal could have been aided by the low sea levels that were present during periods of glaciations. Lessios et al. (1999) found that the *Eucidaris* species from the Atlantic Ocean (PLD ~30 d) had longer distances to cover than could be explained by PLD alone, noting that the distance between Brazil and Ascension is 2300 km, which would

take ~48 days for a larva to travel. They hypothesized that a small island might be used as a "stepping stone along the way." *Tropiometra carinata*'s colonization of St. Helena might have occurred during a glaciation event when sea levels were low, and its range expanded farther out into the Atlantic Ocean than it is presently found.

Alternatively, these two lineages of *T. carinata* might have evolved peripatrically within their own regions. Being a tropical species, *T. carinata* was not likely to have had substantial population declines due to glacial events, but is more likely to have been affected by sea level changes during glacial maximas (Vermeij 1978). The split between lineages within each region could be the result of lowered sea levels during glaciations, which isolated small populations and created a barrier to gene flow, allowing for speciation within a region (Lessios et al. 2001). This may explain why the putative ancestral haplotype is found in Brazil, Madagascar, and the Mozambique Channel.

However, allopatry and peripatry are not the only means by which new species evolve from ancestral species. Sympatric speciation could also explain the genetic patterns observed here. Extrinsic ecological factors have been shown to lead to pre-zygotic isolation within the same habitat (Crow et al. 2010). For example, in a review of the patterns of speciation of marine gastropods, Krug (2011) argued that young sister species are often broadly sympatric and that classical models of allopatric divergence cannot explain these speciation events. He specifically cites microhabitat fidelity, differential habitat choice during mating events, host choice, and substrate selection by larvae as ecological factors that could lead to sympatric speciation. At present, members of *T. carinata* are thought to be generalist filter feeders, with both adults and pentacrinoids being found at different depths and attached to various kinds of substrate (M.D. Correia, pers. obs.). It is possible that the different lineages have evolved due to assortative mating by individuals preferring different microhabitats and resources, but such behavior has yet to be documented. It is, however, unlikely that the Atlantic and Indian Ocean regions would each create two separate lineages with almost identical mutational patterns. Indeed, a more parsimonious explanation for the natural occurrence of the two lineages being found in both the Atlantic and Indian Oceans is that gene flow has occurred during fluctuations in the strength of the Benguela upwelling after its period of intensification during the late Pliocene (Lessios et al. 2003).

It is also possible that the sympatric occurrence of these two lineages is due to human-mediated events. Many marine taxa are known to be transported via ballast water and hull fouling (Williams et al. 1988; Carlton & Geller 1993; Drake & Lodge 2007). Studies surveying the contents of ballast water have found considerably fewer echinoderm larvae than those of other species, with echinoderms not being prevalent on introduction lists (Carlton & Geller 1993; Byrne et al. 1997; Gollasch et al. 2000). Still, the successful colonization and invasion of the sea star *Asterias amurensis* Lütken 1871 are well documented (Secord 2003), and there is evidence to suggest human-mediated translocation of the cosmopolitan brittle star, *Ophiactis savignyi* Müller and Troschel 1842 (Roy & Sponer 2002). Therefore, for an echinoderm to establish itself outside its native range after human-mediated transport is not without precedent. It is important to note that if *T. carinata* is being successfully transported by humans, it may be difficult to fully understand the evolutionary history of this species (Carlton & Geller 1993).

While the means by which *T. carinata* has dispersed are presently unknown, there is evidence to suspect that *T. carinata* may indeed be comprised of multiple species. Previous genetic studies have concluded that similar COI nucleotide divergence as is found in *T. carinata* corresponds to species-level divergence in echinoderms (Landry et al. 2003; Helgen & Rouse 2006). Likewise, a review of COI sequences from 192 species of echinoderms in 125 genera by Ward et al. (2008) revealed that the mean intraspecies divergence of the COI gene between echinoderm species within the same genus was 0.62 ± 0.09%. For crinoids specifically, the mean was 1.30 ± 0.90% and ranged 0–3.04% (Ward et al. 2008). Percent nucleotide distances between some haplotypes of *T. carinata* were similar to that between all the *T. carinata* haplotypes and *T. carinata*'s morphologically distinct sister taxa, *T. macrodiscus* (between 3 and 4%; Table 3). Moreover, a review and meta-analysis by Hart & Sunday (2007) shows that 95% statistical parsimony networks that utilize data from non-recombining loci with rapid lineage sorting rates, such as mtDNA, have a very low false-positive error rate when identifying species boundaries. Indeed, *T. carinata* has been historically recognized as various morphologically distinct species and subspecies throughout its native range (Clark 1921; Messing 2010a). However, morphological plasticity has been noted (Clark 1917), and a recent review has accepted all morphologies containing "aboral arm carination continued to arm tip, ranging from slight to exaggerated; cirri 13–38, of 20–30 segments, 15–27 mm long; arms to 180 mm

(usually no more than 135 mm) long" as *T. carinata* (Messing 2010a). Given our results and those of the aforementioned studies, we conclude that the crinoid specimens found in these regions and accepted as *T. carinata* probably comprise a species complex. However, to definitively determine whether these lineages comprise separate species will require future research to investigate behavioral, morphological, physiological, and additional genetic divergence among the lineages defined here.

Finally, while these lineages appear to adhere to the phylogenetic species concept, in that each lineage is monophyletic, it is unknown whether members of the two lineages are reproductively incompatible, and so also adhere to the biological species concept (Mayr 1942; de Queiroz 1998). Possible barriers to gene flow between broadcast spawning species include spatial differences during gamete release and gametic incompatibility (Levitan et al. 2004). Moreover, asynchronous timing of gamete release even within the same day can be a significant barrier to gene flow (Byrne & Anderson 1994). In his study of Caribbean *T. carinata*, Mortensen (1920) noted that he was able to collect most of his larval specimens during late March and April. The spawning season for *T. carinata* in Brazil is suspected to be between December and March (M.D. Correia, pers. obs.) and the spawning season in other regions is as of yet undocumented. However, such asynchronous spawning times can further advance incipient speciation events.

To conclude, we suggest that future studies investigating diversification within *T. carinata* should include samples from additional populations (such as those in the Caribbean and near India) and also incorporate microsatellite analysis to determine if there is contemporary gene flow between individuals that show high levels of COI divergence. Additional sampling in these locations may help resolve the phylogeny or possibly even reveal more lineages. Likewise, future ecological and behavioral studies of *T. carinata* should attempt to understand the environmental factors that are associated with spawning events. Future studies should also closely examine morphology in comparison with genetic data to determine whether these lineages can be distinguished from one another based on morphological characteristics (i.e., whether they are truly "cryptic species," or simply unrecognized biodiversity). Finally, it is important to determine whether *T. carinata* is capable of dispersing naturally, possibly using tolerance tests, or if their dispersal is being aided by human-mediated events. If these lineages were once naturally separated species and human-mediated movement is responsible for their current sympatry, they may be hybridizing. Wolf et al. (2001) noted that the displacement of pure conspecifics by the hybrids of those species is a conservation concern in many taxa.

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