Lack of Character Displacement in the Male Recognition Molecule, Bindin, in Altantic Sea Urchins of the Genus *Echinometra*

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Bindin, a protein involved in sea urchin sperm–egg recognition and adhesion, is under positive selection in genera with sympatric species but evolves neutrally in genera in which all species are allopatric. This pattern has led to suggestions that reinforcement may be the source of the observed selection. Reproductive character displacement, or increased divergence of reproductive characters in areas where closely related species overlap, is often a consequence of reinforcement and has been shown to be present in one Indo-Pacific species of the genus *Echinometra*. In the Atlantic species of the same genus, positive selection has been shown to act on bindin of *Echinometra lucunter*. To examine whether the source of this selection is reinforcement, we determined variation on the first exon of bindin in *E. lucunter* in the Caribbean, where it is sympatric with *Echinometra viridis*, and in the rest of the Atlantic, where *E. viridis* is absent. There was no differentiation between bindin sequences from the two geographic regions; similar levels of positive selection were found to be acting in both areas. The similarities were not due to gene flow; mitochondrial DNA from the two regions indicates that *E. lucunter* populations most likely originated in the Atlantic and have not exchanged genes with Caribbean populations for approximately 200,000 years. The lack of evidence of stronger selection on bindin of *E. lucunter* in areas of sympatry with its sister species suggests that the source of selection is not reinforcement. Processes acting within species, such as sexual selection, sperm competition, or sexual conflict, are more likely to be involved in the evolution of this molecule.

Introduction

In many free spawning marine organisms, mate recognition can occur on the level of interaction between gametes and is influenced by the action of a small set of molecules. Such molecules often evolve rapidly under strong selection, as indicated by an excess of amino acid replacement substitutions (d_N) compared with silent substitutions (d_S) (Civetta and Singh 1995; Swanson and Vacquier 2002a, 2002b; Swanson et al. 2004). The identification of the source of this selection, however, is not easy (Swanson and Vacquier 2002a). In sea urchins, the best characterized molecule involved in species recognition is the acrosomal protein bindin. Bindin mediates adhesion and fusion of sperm to the egg surface (Vacquier and Moy 1977). Variation in bindin of the sea urchin genus *Echinometra* has been shown to affect species specificity of these interactions (Metz et al. 1994) and fertilization success in intraspecific crosses (Palumbi 1999). Across echinoid genera, bindin divergence is correlated with heterospecific incompatibility in fertilization (Zigler et al. 2005). Bindin has been found to evolve under positive selection in some, but not all, echinoid genera. *Echinometra* (Metz and Palumbi 1996), *Strongylocentrotus* (Biermann 1998), and *Heliocidaris* (Zigler et al. 2003), genera that contain species with sympatric congeners, show a signal of positive selection in the evolution of their bindins. *Arbacia* (Metz et al. 1998) and *Tripneustes* (Zigler and Lessios 2003), genera in which all species are allopatric, do not. The only exception to this pattern is *Lytechinus*, which contains two species with overlapping distributions in the Caribbean with bindins that show no clear evidence of selection (Zigler and Lessios 2004). Even in *Lytechinus*, however, bindin alleles of the two Caribbean species are reciprocally monophyletic, though mitochondrial DNA (mtDNA) is not, which suggests a higher rate of evolution of bindin (Palumbi and Lessios 2005). That only genera with sympatric species show evidence of selection in bindin has led several authors to suggest that reinforcement may be a major source of selection on this molecule (Metz et al. 1998; Swanson and Vacquier 2002b; Palumbi 2009). Others (Zigler and Lessios 2003; McCartney and Lessios 2004; Lessios 2007) have suggested that the pattern is more likely the product of what Templeton (1981) and Noor (1999) have called "differential fusion," that is, the higher probability that species with differentiated reproductive characters can coexist without either fusing or going selectively extinct in sympatry. In cases of differential fusion, the establishment of reproductive isolation occurs before secondary contact, so there is no selection on reproductive traits due to the challenge of sympatric species.

There are several alternate hypotheses as to the nature of selection operating on bindin, which are independent of the challenge by a related species (Metz et al. 1998). Such intraspecific forces include sexual conflict, sperm competition, and sexual selection. Polyspermy, a lethal condition for the developing embryo, is a problem even under sperm limiting conditions in *Evechinus chloroticus* (Franke et al. 2002), indicating that this could be a major source of selection on reproductive traits in some species. Several authors have suggested that avoidance of polyspermy could create sexual conflicts in egg–sperm interactions (Galindo et al. 2003; Haygood 2004; Levitan 2004; Levitan and Ferrell 2006; Levitan et al. 2007). Experiments by Levitan and Ferrell (2006) showed that in *Strongylocentrotus franciscanus*, there is an interaction between sperm density and genotype frequency of bindin alleles; when sperm is limited, males and females with matching bindin alleles have higher fertilization success, but when sperm densities are high, offspring of males and females with divergent bindin genotypes survive at greater rates. Assortative mating on

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the basis of bindin genotype has been observed in *Echinometra mathaei* (Palumbi 1999). Thus, the importance of intraspecific forces, such as sexual conflict and sexual selection, on the evolution of bindin is supported by experimental evidence.

In one Indo-Pacific species, *Echinometra oblonga*, a clear pattern of reproductive character displacement (RCD) suggests that reinforcement does play a role in the rapid evolution of bindin (Geyer and Palumbi 2003). In localities at which *E. oblonga* coexists with *E.* sp. C, it has bindin alleles much more divergent from those of *E.* sp. C than in localities where this congener is absent. Other Indo-Pacific species of *Echinometra*, however, also show evidence of strong selection, even where there are no clear geographic patterns to indicate character displacement (Metz and Palumbi 1996). If character displacement were found in other pairs of *Echinometra* species with sympatric and allopatric populations, the inference that reinforcement is the source of selection in bindin evolution would be strengthened (Riginos and McDonald 2003). Conversely, its absence would make the case for reinforcement less likely (Riginos et al. 2006). We, therefore, looked for geographic variation in the bindin molecule of two Atlantic species of *Echinometra* with partially overlapping geographical distributions.

Two species of *Echinometra* coexist in the Caribbean. *Echinometra viridis* is restricted to this Sea, whereas *E. lucunter* is spread on both sides of the tropical Atlantic, ranging from Dakar to Angola on the African coast, and from Bermuda to Florianopolis, Brazil, on the American shores. It is also the only species of *Echinometra* found in the central Atlantic islands of Ascension and St Helena (Mortensen 1943). The common Atlantic stock was separated from the eastern Pacific species, *E. vanbrunti*, by the Isthmus of Panama about 3 million years ago (Ma), then split into the two morphologically distinct Atlantic species about 1.5 Ma (McCartney et al. 2000). *Echinometra lucunter* eggs will not permit fertilization by either *E. viridis* or *E. vanbrunti* sperm, although its sperm can fertilize eggs of the other two species at rates only slightly lower than its own eggs (Lessios and Cunningham 1990; McCartney and Lessios 2002). Despite this one-way isolation, extensive isozyme (Lessios 1979, 1981a; Bermingham and Lessios 1993), mtDNA (Bermingham and Lessios 1993; McCartney et al. 2000), and bindin (McCartney and Lessios 2004) sampling has never identified a hybrid among postmetamorphic sea urchins in the Caribbean, which suggests that reproductive isolation in nature is complete. Such complete isolation is likely to arise from postzygotic isolating barriers, because the annual reproductive cycles of the two sympatric species overlap (Lessios 1981b) and neither shows a lunar rhythm in spawning (Lessios 1991), leaving few alternatives as possible prezygotic barriers. Thus, it is possible that selection to avoid hybridization is operating on the two sympatric species in the Caribbean.

McCartney and Lessios (2004) found evidence that the bindin of *E. lucunter* (but not of *E. viridis*) evolves under strong selection. As *E. lucunter* is also the species in which eggs are incompatible with heterospecific sperm, the evolution of its bindin appears to be tracking changes in the egg receptor. Unfortunately, the sea urchin bindin receptor EBR1 is a molecule so large (4,595 amino acids) that its variation cannot be readily studied in the same manner as bindin (Kamei and Glabe 2003). Although McCartney and Lessios (2004) suggested a number of alternative sources of selection on *E. lucunter* bindin, their samples included only Panamanian populations, leaving reinforcement as a possibility. In the present study, we analyze variation of the most variable section of *E. lucunter* bindin from Caribbean populations, where it is sympatric with *E. viridis*, and from Atlantic populations, where it is free of the challenge of this congener, to determine whether there is any evidence of higher bindin divergence in sympatry than in allopatry.

Table 1
Number of Individuals Sampled and of Unique Bindin Alleles Encountered in *Echinometra lucunter* at Localities within and without the Caribbean Sea

Region	Locality	No. of Individuals	No. of Unique Alleles
Atlantic	Tamandaré, Brazil	10	12
	Rio de Janeiro, Brazil	6	9
	Salvador, Brazil	9	13
	Ascención, Central Atlantic	7	9
	St. Helena, Central Atlantic	9	13
	São Tomé, Eastern Atlantic	6	8
	Dakar, Senegal, Eastern Atlantic	4	7
	Turtle Bay, Bermuda	7	11
	Fort Pierce, Florida	2	3
	Total	60	85
Caribbean	Caribbean coast of Panama	33	33
	Boca Chica, Dominican Rep.	6	10
	Carrie Bow Cay, Belize	10	15
	Discovery Bay, Jamaica	9	14
	San Salvador, Bahamas	5	15
	Total	63	87

Materials and Methods

Sampling

We sampled a total of 124 individuals of *E. lucunter* from five populations in the Caribbean Sea, where it is sympatric with *E. viridis,* and from nine populations in the Atlantic Ocean, where *E. viridis* is absent. (table 1, fig. 1). The Caribbean sample includes sequences from Panama that were previously obtained by McCartney and Lessios (2004), with GenBank accession numbers AY451242–AY451275. Thirty-one additional bindin sequences of *E. viridis* and 16 of *E. vanbrunti* (accession numbers AY451276–AY451323, McCartney and Lessios 2004) were included in the analyses. New sequences have been deposited in GenBank under accession numbers GQ231594–GQ231731.

Sequencing

Genomic DNA was extracted from gonad tissue preserved in dimethyl sulfoxide–high salt buffer (Seutin et al. 1991) according to methods described in Lessios et al. (1996). We amplified an 840- to 950-bp fragment of the bindin molecule corresponding to the first exon and approximately

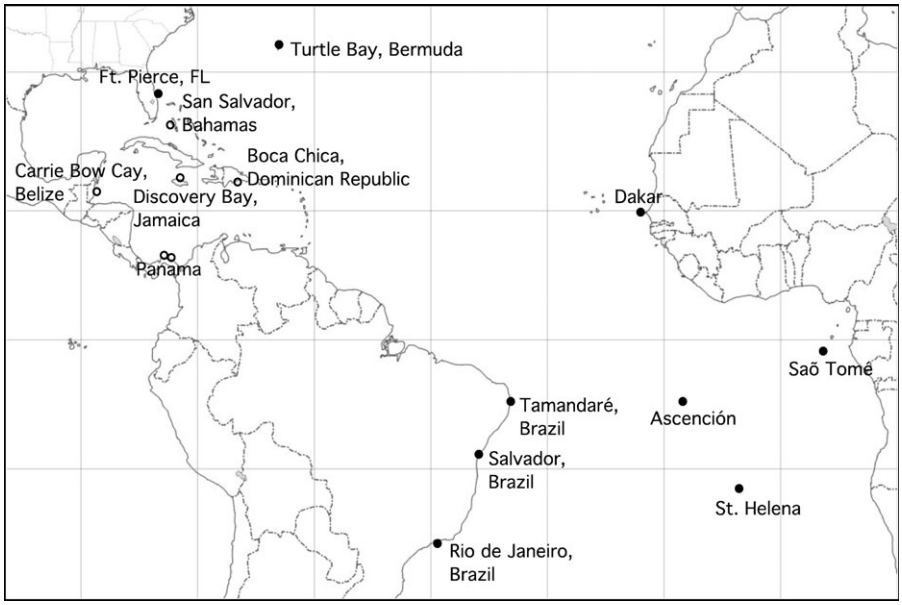

FIG. 1.—Collection localities of *Echinometra lucunter*. Open circles mark populations sympatric with *Echinometra viridis*, filled circles mark allopatric populations.

470–490 bp of the bindin intron using primers BGEN F2 (5′-AACTACCCCCAAGCCATGAATC-3′) and MB1136-(5′-ARGTCAATCTTSGTSGCACC-3′). The first exon is the region of the bindin molecule in which most of the variation is found, and where evidence of selection has been demonstrated (Metz and Palumbi 1996; Landry et al. 2003; McCartney and Lessios 2004), and for this reason it is the segment of bindin usually analyzed in assessments of intraspecific variation of the molecule (Metz and Palumbi 1996; Palumbi 1999; Geyer and Palumbi 2003, 2005; Landry et al. 2003). Amplicons were cloned using the pGEM-T Easy Vector System (Promega). Five clones per individual were sequenced using the BigDye Terminator v3.1 cycle sequencing system (Applied Biosystems) on a 3130 Genetic Analyzer sequencer (Applied Biosystems). Consensus sequences of at least three clones per allele were constructed in order to reduce amplification and cloning errors. If sequences from all five clones of an individual matched each other, the individual was considered a homozygote and was counted as two identical sequences of bindin. Additional clones were sequenced on an ad hoc basis when errors and ambiguities could not be resolved by majority rule or when the differences indicated the presence of a second allele, for which a new consensus sequence was obtained by additional cloning.

Alignment

Sequences were aligned with Sequencher v. 4.6 (Gene Codes Corporation). Length variation in the first exon of the bindin of *Echinometra* complicates alignments of this section. The variable length region contains two to eight repeats with the predicted amino acid sequence AX-AXPXGX, each separated by two to five Glycine residues (fig. 2). High numbers of insertions and deletions and similarity among repeats can cause uncertainties as to positional homology of the repeat and the poly-Glycine segment. Misalignments can artificially increase estimated replacement rates and apparent homoplasy. To minimize these problems, sequences were aligned by eye in order to retain repeats as complete units and to add gaps that reduce apparent nucleotide differences. Poly-Glycine segments were arbitrarily aligned to the 3′ end of each associated repeat unit.

Phylogenetic Analysis

To reconstruct the genealogy of unique sequences, the most appropriate model of molecular evolution was chosen as one that minimized Akaike′s (1974) Information Criterion using Modeltest v.3.7 (Posada and Crandall 1998). The best fit model was that of Kimura (1981) with a γ correction ($\alpha = 1.26$). Using this model, we estimated a Neighbor-Joining (NJ) tree in PAUP* 4.0b10 (Swofford 2001); the tree was rooted on 13 sequences of 3 Indo-West Pacific *Echinometra* species (GenBank accession numbers U39502–U39514). Alignment gaps were treated as missing data for affected pairwise comparisons. Statistical support for the topology was obtained by bootstrapping in 1,000 iterations. Maximum likelihood (ML) analyses were also performed using GARLI 0.951-1 (Zwickl 2006; http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html) estimating all parameters from the data under the general time reversible model with a γ correction ($\alpha = 1.28$). The ML tree was bootstrapped in 500 iterations.

Arlequin 3.11 (Excoffier et al. 2005) was used to calculate population statistics and to perform Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992), based on Kimura's (1980) two-parameter model of molecular evolution with significance estimated using 10,100 permutations of alleles and localities. Molecular diversity, based on Kimura two-parameter distance, was calculated

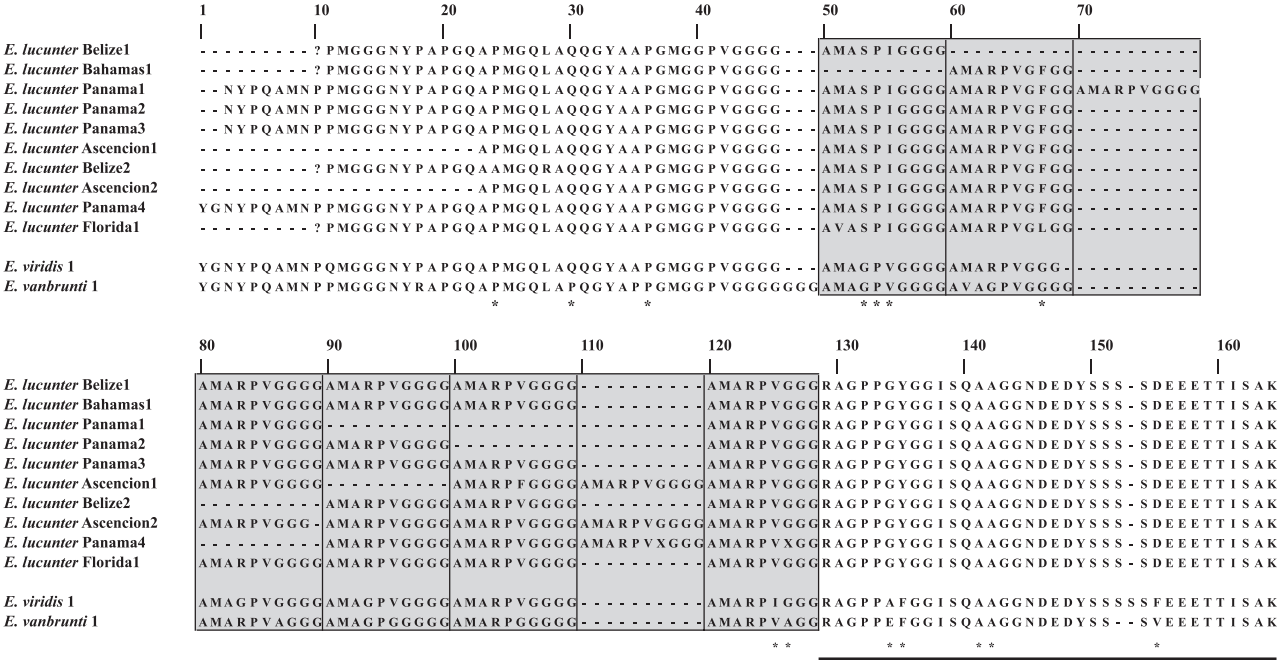

FIG. 2.—Alignment of amino acid sequences of selected bindin alleles of *Echinometra lucunter*, *Echinometra viridis*, and *Echinometra vanbrunti*. Amino acid alignment is based on nucleotide variation and results in gaps among the AXAXPXGX repeats (repeat area is shaded, each repeat enclosed in a box). Asterisks at the bottom identify sites under positive selection, according to ML analyses (see table 6). The solid line under the alignment indicates a hypervariable region.

in Mega 3.1 (Kumar et al. 2004) with alignment gaps treated as missing data for affected pairwise comparisons. Haplotype diversity was calculated in DNAsp 4.50.2 (Rozas and Rozas 1999) with all sites containing gaps excluded from the analysis. Recombination was estimated using the four gamete test (Hudson and Kaplan 1985) and the recombination parameter, R (Hudson 1987) as implemented in DNAsp 4.50.2. To reconstruct the history of colonization of *E. lucunter*, TCS v. 1.21 (Clement et al. 2000) was used for the construction of a statistical parsimony (Templeton et al. 1992) network of Cytochrome Oxidase I (COI) haplotypes of data taken from McCartney et al. (2000) (GenBank Accession numbers AF255468–AF255510) with the confidence of connection limits set at 95%.

Tests for the Presence of Selection

McDonald–Kreitman (1991) tests of selection were performed using DNAsp 4.50.2 (Rozas and Rozas 1999). The ratio of amino acid replacement (d_N) and silent (d_S) substitutions per site was estimated in Mega 3.1 (Kumar et al. 2004) using the Pamilo and Bianchi (1993) and Li (1993) method. The significance of the excess in replacement substitutions was tested in pairwise Fisher's Exact tests using the modified Nei and Gojobori (1986) method as described in Nei and Kumar (2000) and implemented in MEGA 3.1, with the transition/transversion ratio estimated from the data ($R = 0.955$). We also conducted tests for selection in the Codeml module of PAML 3.15 (Yang 1997). For this analysis, an initial, unrooted, NJ tree (without the outgroup Indo-Pacific species of *Echinometra*) was generated in PAUP* using only unique bindin sequences and eliminating ambiguously aligned codons. Only the first two and the last one repeat of the first exon were included, because they were present in almost all sequences of *E. lucunter* and could be unambiguously aligned. Two codons at the 3′ end of the first exon (corresponding to positions 152 and 153, fig. 2) were excluded because they could not be unambiguously aligned between species. Also excluded were four sequences of *E. lucunter* and one of *E. viridis* because they had large ($\geq$24 bp) gaps that could introduce error into the analysis. The resulting alignment consisted of 87 amino acids and included several small ($<$6 bp) unambiguously aligned gaps that were shared by no more than two sequences, as recommended by Yang (1997). This alignment was subjected to analysis of the distribution of the ratio of amino acid replacement to silent substitutions (ω) among sites and among branches.

We analyzed variation of ω among amino acid sites of the first exon using site-specific models described in Yang (1998), Yang et al. (2000), and Wong et al. (2004). As null models for variation between sites, we used the neutral one-ω model (M0), the nearly neutral (M1a), and the β distribution model (M7). As models that allow selection, we used model M2a, discrete models with either two (M3 $k = 2$) or three (M3 $k = 3$) site classes of ω, and the $\beta + \omega$ model (M8), which allows for a continuous distribution of ω values across sites. We also used lineage-specific models to assess selection along specific phylogenetic branches. We compared the likelihood of a model that allows one ω ratio for all branches (1ω) with one that allows for a separate ratio for each species branch (3ω). We further used branch-sites models (Yang and Nielsen 2002; Yang et al. 2005; Zhang et al. 2005) for a simultaneous examination of variation in selection among amino acid sites and among lineages of bindin. Model MA1

Table 2
Molecular (π) and Haplotype (Hd) Diversity of Bindin and COI in Neotropical Species of *Echinometra*

	Bindin[a]		COI[b]	
	π[c]	Hd	π[c]	Hd
Echinometra lucunter Atlantic	0.003	0.421	0.005	0.858
E. lucunter Caribbean	0.003	0.457	0.008	0.883
E. lucunter all localities	0.003	0.333	0.008	0.859
Echinometra viridis	0.008	0.833	0.009	0.800
Echinometra vanbrunti	0.007	0.524	0.008	0.758

[a] Data from this study and from McCartney and Lessios (2004).
[b] Data from McCartney et al. (2000).
[c] Based on Kimura's two-parameter distance correction.

assumed that d_N/d_S ratios for all background branches (ω_0) varied between 0 and 1, whereas the foreground ratio was free to vary and was compared with the nearly neutral model M1a. This test can produce significant results if there is relaxation of constraints, rather than positive selection, in the foreground branch. Model MA2 is similar to MA1, but uses as the null model MA1 with the foreground $\omega = 1$, and is thus considered a direct test of positive selection (Zhang et al. 2005). Model MB allows all ω parameters to be estimated from the data, rather than constraining them, and so is the most general branch-sites model.

Results

Genetic Diversity

We obtained 120 bindin alleles (85 unique ones) from 60 individuals of *E. lucunter* from 9 populations in the Atlantic, and 126 alleles (87 unique ones) from 63 individuals in 5 populations in the Caribbean, where this species is sympatric with its sister species, *E. viridis* (table 1). The data from previously unsampled localities indicate that the finding of McCartney and Lessios (2004) from Panamanian populations, that molecular and haplotype diversity in the bindin of *E. lucunter* is lower than that of other neotropical species of *Echinometra*, holds true for the entire range of this species (table 2). There is no concomitant reduction in the diversity of COI as would have been expected if the lower diversity in bindin were due to a historical demographic factor, such as a genetic bottleneck (table 2). There are no obvious differences in bindin molecular diversity between populations inside or outside the Caribbean.

Bindin Gene Genealogy

Reconstructions of the bindin gene genealogy of *Echinometra* by NJ and ML converged on similar topologies, differing only in the details of the arrangements of the terminal branches. Because none of the nodes in which the two trees differed had strong bootstrap support, only the NJ phylogram is presented (fig. 3). Our genealogy, based on many alleles but only the first exon of bindin, is not entirely consistent with that of McCartney and Lessios (2004), based on fewer alleles but incorporating the entire molecule. Both phylogenies show bindin alleles of each Neotropical species of *Echinometra* clustered into reciprocally monophyletic units, but in the McCartney and Lessios (2004) phylogeny, the sister clade of *E. lucunter* alleles consisted of alleles of *E. vanbrunti*. In both phylogenies, the bootstrap support of the basal node of the three species is weak, so the species-level phylogeny of bindin is best considered as a tritomy. This topology differs from that of the mitochondrial COI gene (McCartney et al. 2000), which shows a well-supported sister relationship between *E. lucunter* and *E. viridis*, with *E. vanbrunti* as an outgroup. Low levels of recombination ($R = 0.001$) were estimated for the first exon of bindin in these three species, analyzed according to the method of Hudson (1987), and only four recombination events were detected via the four gamete test (Hudson and Kaplan 1985). Separate analyses based on each recombination block produced phylogenetic trees with little bootstrap support for any node. Thus, possible distortion of the gene genealogy due to recombination is not so great as to lead to incorrect conclusions regarding selection. In the genealogy of the first exon of bindin shown in figure 3 there was no support for any subclades within *E. lucunter*, nor any indication of phylogenetic separation of alleles where it is sympatric with *E. viridis* and where it is not. Indeed, five alleles were shared between the Caribbean and the Atlantic (fig. 3). Thus, there is no indication that different bindin alleles predominate in the region of overlap between *E. lucunter* and *E. viridis*.

Intraspecific Differentiation

Overall divergence in the first exon of bindin between Caribbean and Atlantic populations of *E. lucunter*, as measured by Kimura's two-parameter distance, was equal to mean divergence between populations within each of these regions (table 3). AMOVA also indicated that the geographic distribution of bindin is not different from random ($P = 0.27$) and that 102.19% of the variation was between individuals within populations. The Φ_{CT} value between regions was -0.06.

Pairwise F_{ST} values (table 4) of bindin of *E. lucunter* were large and significant between the Atlantic island of Ascención and a number of other populations, including all of the Caribbean populations except Belize (table 4). This, however, is not indicative of regional differences, because F_{ST} values were larger and also significant in comparisons between Ascención, on the one hand, and the Atlantic populations of São Tomé, Bermuda, and all three populations in Brazil, on the other, indicating that Ascención is genetically isolated. All other comparisons between Atlantic and Caribbean populations of *E. lucunter* produced F_{ST} values that were very small and not different from random. Thus, there was no evidence of differentiation of bindin between populations that are sympatric and populations that are allopatric with *E. viridis*. Given this homogeneity within *E. lucunter*, it is not surprising that there was also no indication that bindin of *E. lucunter* was more divergent from that of *E. viridis* in the Caribbean than in the Atlantic (table 3), as would have been expected from RCD.

Selection

There was no significant excess of amino acid replacement substitutions relative to silent ones in the entire first exon of bindin either within *E. lucunter* or in comparison to

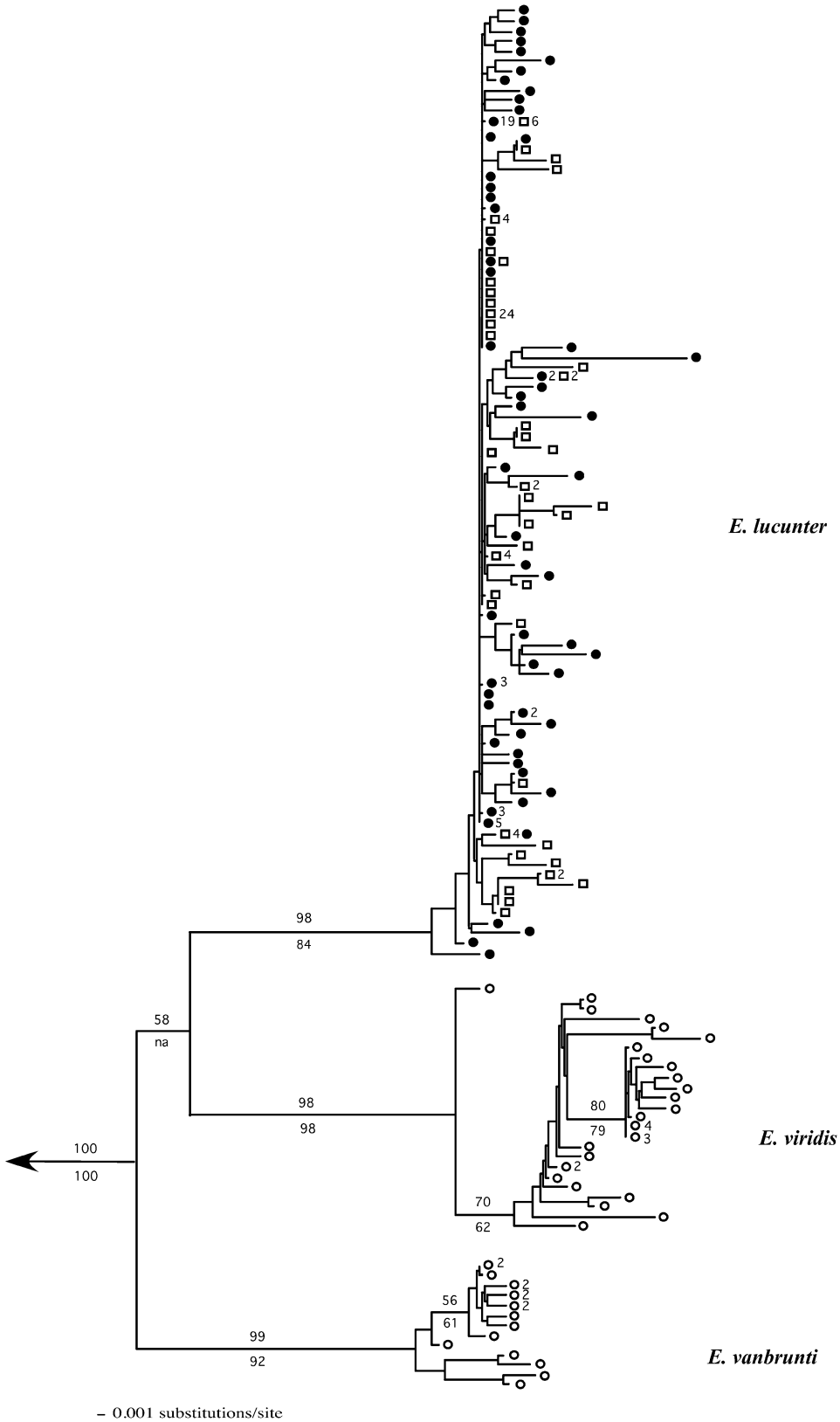

FIG. 3.—Gene genealogy of bindin alleles of *Echinometra* from the New World and the Atlantic Ocean. Genealogy was constructed by NJ based on Kimura (1981) distance with a γ correction and was rooted on sequences from three species of *Echinometra* from the Indo-West Pacific. Alleles of *Echinometra lucunter* found in the Caribbean are represented by filled circles, those in the Atlantic with open squares. Numbers next to symbols indicate multiple occurrences of indistinguishable alleles. Numbers above branches indicate bootstrap support from 1,000 iterations in NJ. Numbers below branches indicate bootstrap support from 500 iterations in ML. Bootstrap support is not shown for nodes uniting only terminal axa.

Table 3
Synonymous (d_S), Nonsynonymous (d_N) Proportions of Substitutions, and Mean Kimura Two-Parameter Distance (K_2) in the First Exon of Bindin

	d_N[a]	d_S[a]	d_N/d_S[b]	K_2
Echinometra lucunter all	0.0029	0.0037	0.78	0.003
E. lucunter Atlantic	0.0034	0.0025	1.38	0.003
E. lucunter Caribbean	0.0024	0.0046	0.52	0.003
Echinometra viridis	0.0067	0.0120	0.55	0.008
Echinometra vanbrunti	0.0067	0.0071	0.95	0.007
E. lucunter Atlantic to *E. lucunter* Caribbean	0.0036	0.0030	0.84	0.003
E. viridis to all *E. lucunter*	0.0447	0.1142	0.39	0.062
E. viridis to *E. lucunter* Atlantic	0.0465	0.1210	0.38	0.065
E. viridis to *E. lucunter* Caribbean	0.0433	0.1091	0.40	0.059
E. viridis to *E. vanbrunti*	0.0491	0.0988	0.50	0.064
E. lucunter to *E. vanbrunti*	0.0688	0.0720	0.96	0.071

[a] Pamilo and Bianchi (1993) and Li (1993) method.
[b] d_N/d_S is not significantly >1 in any pairwise comparison (Fisher's Exact Tests).

E. viridis (table 3). There was also no indication in the average rate of the two types of substitutions that there is stronger selection on bindin in the area of geographic overlap between the species. McDonald–Kreitman tests found no significant excess of fixed versus. polymorphic nonsynonymous differences between *E. lucunter* and *E. viridis*, whether the comparison involved all samples, or just those from the region of geographic overlap (table 5). However, average rates of substitution over an entire sequence are incapable of detecting positive selection that acts only on specific amino acid sites. We therefore relied on the ML methods of Yang (1998), Yang et al. (2000), and Yang and Nielsen (2002) to ask whether the expanded geographic coverage of the present study relative to that of McCartney and Lessios (2004) could still identify positive selection.

Because the ML models are designed to detect selection along specific branches of a gene genealogy, and because bindin alleles of *E. lucunter* do not sort out phylogenetically according to geographic area, we were obliged to carry out an analysis that included the first exon of bindin variation of all populations. Several of the discrete site-specific models (Yang 1998; Yang et al. 2000) identified codons with elevated ω, but only the M8 model was significantly more likely than its null comparison M7 (tables 6 and 7). Although 13% of the codons were identified as possibly being under selection in this analysis ($\omega = 4.4$; table 6), none had a significant (>95%) posterior probability of belonging to that class of sites. Similarly, the branch-specific model (Yang and Nielsen 2002), which allowed for separate values of ω for each species branch, was not significantly better than the null model (tables 6 and 7). We constructed nine branch-sites models (Yang and Nielsen 2002; Zhang et al. 2005), each of which allowed the ancestral branch of all the alleles of each species to act as the foreground branch, and to differ from the background rate. The models with *E. viridis* or *E. vanbrunti* bindins as the foreground (data not shown) produced results identical to those of McCartney and Lessios (2004) in that they found no positive selection along these branches. The models with *E. lucunter* bindin as the foreground branch, on the other hand, showed evidence for positive selection. Model A1 (table 6), which forced the background branches to have $\omega = 1$, while letting the foreground branches vary, was significantly different from the null (table 7). We also tested this model against one (MA2) in which the foreground branches were forced to have $\omega = 1$. This comparison was also significant (table 7), indicating that the signal is caused by positive selection, and not simply relaxation of purifying selection. Model MB indicated several classes of sites with extremely high values of ω (table 6) and was significantly better than the null model (table 7), but failed to identify which sites were under positive selection. The inability to identify specific sites under selection may have been caused by the short length of the sequence, which decreases power (Anisimova et al. 2001), or by the extreme estimated parameter values, which may have resulted in the exclusion of all sites.

History of Colonization

Reinforcement would be more likely if *E. lucunter* and *E. viridis* diverged in allopatry and then came into secondary contact than if they speciated sympatrically, or if they

Table 4
Pairwise F_{ST} Values at Bindin among Populations of *Echinometra lucunter* in which more than Three Individuals Were Sampled

	Dominican Republic	Jamaica	Bahamas	Panama	Belize	Sao Tomé	St. Helena	Ascención	Dakar	Tamandaré, Brazil	Salvador, Brazil	Rio, Brazil
Jamaica	−0.048	—										
Bahamas	0.003	0.008	—									
Panama	−0.102	−0.079	−0.048	—								
Belize	0.006	−0.002	0.019	−0.083	—							
São Tomé	−0.034	−0.055	−0.006	−0.143	0.009	—						
St. Helena	−0.002	−0.002	0.033	−0.097	0.061	0.087	—					
Ascención	0.235	0.170*	0.188*	0.107	0.214*	0.255*	0.035	—				
Dakar	−0.068	−0.077	−0.051	−0.146	−0.011	0.047	−0.014	0.163	—			
Tamandaré,Brazil	−0.015	−0.075	0.002	−0.134	0.057	0.045	0.121	0.322*	0.062	—		
Salvador, Brazil	−0.045	−0.071	−0.012	−0.136	0.058	0.048	0.120	0.338*	0.064	0.032	—	
Rio, Brazil	0.001	−0.079	−0.021	−0.141	0.039	0.029	0.092	0.287*	0.019	0.011	−0.004	—
Bermuda	0.015	−0.049	−0.008	−0.096	0.011	−0.029	0.029	0.268*	−0.079	−0.026	−0.095	0.000

*Significant after sequential Bonferroni correction at $\alpha = 0.05$ (Rice 1989) based on 10,100 permutations.

Table 5
McDonald–Kreitman Tests for Selection on the First Exon of Bindin in *Echinometra lucunter*

Geographic Region	Fixed Differences[a]		Polymorphisms[a]		
	Nonsynonymous	Synonymous	Nonsynonymous	Synonymous	P^b
All populations	3	4	18	5	0.153
Atlantic	4	4	16	7	0.405
Caribbean	3	5	15	5	0.091

[a] *Echinometra viridis* was used as the outgroup.
[b] Two-tailed Fisher's Exact Test.

spent a great deal of time in complete sympatry before assuming their current pattern of partial spatial overlap. Because of the possibility of selection, bindin cannot be used to reconstruct the phylogeographic history of these species. Variation in COI, on the other hand, is likely to be selectively neutral. Statistical parsimony analysis indicates that the presumed ancestral haplotype of the existing COI sequences is found only outside the Caribbean (fig. 4), and thus that it is likely that *E. lucunter* originated in the Atlantic, then came into sympatry with *E. viridis* in the Caribbean.

Discussion

Our extensive sampling of the first exon of bindin over the entire species range has confirmed the finding of McCartney and Lessios (2004) from Panamanian populations that selection is acting on this molecule in *E. lucunter*. By all indications, this selection is not limited to the area of sympatry with *E. viridis*, but is a characteristic of the evolution of this molecule in all populations on both sides of the Atlantic Ocean, and in the isolated islands of Ascención and St. Helena. There is no evidence of differentiation between bindin alleles from the Caribbean and the Atlantic, no evidence of higher divergence from alleles of *E. viridis* in the area of sympatry, and thus no pattern of character displacement on the first exon of *E. lucunter*.

Does the absence of character displacement in the bindin of *E. lucunter* indicate that reinforcement is not the selective force acting on this molecule? Reinforcement does not always create a pattern of character displacement (Howard 1993; Lemmon et al. 2004). In order to conclude that the absence of RCD in *E. lucunter* bindin is evidence against reinforcement as a source of the demonstrated selection, it is necessary to consider possible ways in which reinforcement could still be involved without resulting in differences between the area of sympatry and the area of allopatry. It is possible that 1) gene flow between the two areas homogenizes their bindin allele frequencies or that 2) the similarities between populations in the two areas are a remnant of reinforcement that occurred during previously complete overlap between the ranges of the two species.

1. One possible cause of the lack of differences in bindin of *E. lucunter* between the Caribbean and the Atlantic would be gene flow from the area of sympatry toward the area of allopatry. This hypothesis, however, would be contradicted by the evidence from mtDNA that these regional populations have not exchanged mitochondrial genes for approximately 200,000 years. COI haplotypes of Caribbean populations are monophyletic and nested among haplotypes of Atlantic populations. The F_{ST} value between haplotypes of the two regions (0.37) is high (McCartney et al. 2000). Thus, it is more likely that the lack of regional differentiation of bindin, as in other nuclear genes, is the result of slower evolution of nuclear genes relative to that of mitochondrial genes (Moore 1995; Palumbi et al. 2001), or that the source of selection on bindin both inside and outside the Caribbean is the same. If so, selection could not be due to on-going reinforcement.
2. The absence of character displacement would also not necessarily indicate lack of reinforcement if *E. lucunter* and *E. viridis* arose sympatrically, or if they spent a great deal of time in sympatry before the former expanded its range into areas of allopatry (Howard 1993; Servedio 2004). According to this hypothesis, the bindin constitution of *E. lucunter* could have been shaped by reinforcement between 1.5 Ma when the speciation event occurred (McCartney et al. 2000) and 0.2 Ma, when gene flow between Atlantic and Caribbean populations was interrupted. mtDNA evidence is not consistent with such a hypothesis. The COI genealogy of *E. lucunter* (fig. 4) indicates that the oldest haplotypes are found in the Atlantic but not in the Caribbean. In addition, fossil evidence from Angola indicates that *E. lucunter* was present in the eastern Atlantic during the Pleistocene (Darteville 1953). Although these lines of evidence are not definitive, the most parsimonious explanation is that *E. lucunter* originated in the Atlantic and only later spread to the Caribbean into sympatry with *E. viridis*. Recent secondary sympatry greatly detracts from the possibility of "reinforcement in times past."

Reinforcement is expected to occur when populations develop postzygotic isolation in allopatry, then become sympatric and perfect prezygotic isolation as the result of selection against hybridization (Dobzhansky 1940). "Speciation by reinforcement" would only occur if reproductive barriers have not been completed in allopatry (Noor 1999; Coyne and Orr 2004), but selection for reinforcement could continue to operate to perfect prezygotic isolating barriers between sympatric species even after postzygotic isolation (and thus speciation) is complete. Postmetamorphic hybrids between *E. lucunter* and *E. viridis* have not been found

Table 6
ML Models of ω[a] Variation in Bindin

Model[b]	ℓ[c]	pa[d]	d_N/d_S	Parameter Estimates	Positively Selected Sites[e]
Site-specific models					
M0 (one ratio)	−985.120	1	1.090	$\omega = 1.090$	Not allowed
M1a (nearly neutral)	−985.197	1	1.000	$p_0 = 0.444$	Not allowed
M2a (selection)	−983.201	3	1.176	$p_0 = 0.910$, $p_1 = 0.000$ ($p_2 = 0.090$) $\omega_2 = 4.401$	24P, 67F, 134G
M3 (discrete) $k = 2$	−983.882	3	1.176	$p_0 = 0.910$ ($p_1 = 0.090$) $\omega_0 = 0.858$, $\omega_1 = 4.398$	24P, 67F, 134G
M3 (discrete) $k = 3$	−984.674	5	1.184	$p_0 = 0.391$, $p_1 = 0.513$ ($p_2 = 0.096$) $\omega_0 = 0.854$, $\omega_1 = 0.854$ $\omega_2 = 4.278$	24P[f], 67F, 134G
M7 (beta)	−986.810	2	1.000	$p = 1.514$, $q = 0.005$	
M8 (beta & ω)	−983.202	4	1.177	$p_0 = 0.911$ ($p_1 = 0.089$) $p = 99.000$, $q = 15.942$, $\omega = 4.423$	24P, 30Q, 36P, 54S, 55P, 69F, 125V, 126G, 134G, 141A, 142A
Branch-specific models					
One ratio (ω)	−985.197	1	1.000	$\omega = 1.000$	
Three ratio (3ω)	−984.082	4	1.129	$\omega_0 = 1.1293$, $\omega_1 = 1.7744$, $\omega_2 = 0.2417$	
Branch-sites models					
Model A1	−981.994	3	0.499	$p_0 = 0.024$, $\omega_{back} = 1$, $\omega_{for} = 1$ $p_1 = 0.925$, $\omega_{back} = 1$, $\omega_{for} = 1$ $p_{2a} = 0.001$, $\omega_{back} = 1$, $\omega_{for} = 160.441$ $p_{2b} = 0.050$, $\omega_{back} = 1$, $\omega_{for} = 160.441$	54S, 56I, 69F, 134G, 135Y, 155D
Model A2	−985.310	3	1.000	$p_0 = 0.464$, $\omega_{back} = 1$, $\omega_{for} = 1$ $p_1 = 0.306$, $\omega_{back} = 1$, $\omega_{for} = 1$ $p_{2a} = 0.138$, $\omega_{back} = 1$, $\omega_{for} = 1$ $p_{2b} = 0.091$, $\omega_{back} = 1$, $\omega_{for} = 1$	None
Model B	−980.174	5	0.529	$p_0 = 0.916$, $\omega_{back} = 0.899$, $\omega_{for} = 0.899$ $p_1 = 0.033$, $\omega_{back} = 6.166$, $\omega_{for} = 6.166$ $p_{2a} = 0.049$, $\omega_{back} = 0.899$, $\omega_{for} = 169.435$ $p_{2b} = 0.002$, $\omega_{back} = 6.166$, $\omega_{for} = 169.435$	None

[a] Ratio of nonsynonymous to synonymous substitutions.
[b] Model designations follow Yang and Nielsen (2002), Wong et al. (2004), and Yang et al. (2005).
[c] Log-likelihood values.
[d] Number of parameters.
[e] Amino acid (AA) sites under positive selection. Numbers refer to AA position in alignment (fig. 2). Letters refer to reference AA in first sequence of alignment.
[f] Bayes Empirical Bayes posterior probability ≥95%.

(McCartney et al. 2000), yet prezygotic isolation is still asymmetrical and incomplete (Lessios and Cunningham 1990; McCartney and Lessios 2002), so hybrid zygotes between the two species are probably still being produced but fail to reach adulthood. Thus, an expectation of reinforcement within the Caribbean is not unreasonable, and neither is the expectation of RCD sensu Butlin (1995), that is, a geographical pattern of differential selection against hybridization after speciation is complete. That no such pattern was revealed and that the probable geographic history of speciation involves an initial period in allopatry suggests that the selective force on bindin of *E. lucunter* has not been reinforcement.

Table 7
Log-Likelihood Ratio Tests Comparing Models Allowing Positive Selection with Their Null Alternatives

Models Compared	$2\Delta\ell$[a]	df[b]	P[c]
Variable sites			
M1 versus M2	−3.992	2	0.136
M1 versus M3 ($k = 2$)	−2.630	2	0.269
M1 versus M3 ($k = 3$)	−1.046	4	0.903
M7 versus M8	−7.215	2	0.027
Variable lineages			
1ω versus 3ω	−2.230	3	0.526
Branches/sites			
M1a versus MA1	−6.406	2	0.040
MA2 versus MA1	−6.633	1	0.010
M3 ($k = 2$) versus MB	−7.415	2	0.025

[a] Log-likelihood ratio.
[b] Degrees of freedom.
[c] Probability derived from the χ^2 distribution.

If reinforcement is not a likely source of selection on bindin, then what are the alternative hypotheses that could explain the signature of positive selection on the bindin of *E. lucunter*? Intraspecific forces such as sexual conflict, sperm competition, and sexual selection could play a role. McCartney and Lessios (2004) have suggested that *E. lucunter*, because it is found in high point population densities almost exclusively in a high energy narrow intertidal zone, is likely to spawn under conditions of high density of mixed sperm. If so, polyspermy, sperm competition, and sexual selection would be more important in this species than they are in *E. viridis* or in *E. vanbrunti*. This hypothesis could explain why the bindin of *E. lucunter* is under positive selection, whereas that of the other two species is not (Levitan and Ferrell 2006). Levitan and Ferrell (2006) demonstrated that crosses between males and females of *S. franciscanus* with divergent bindin alleles increase in frequency when sperm densities are high, which would suggest that there is frequency-dependent selection

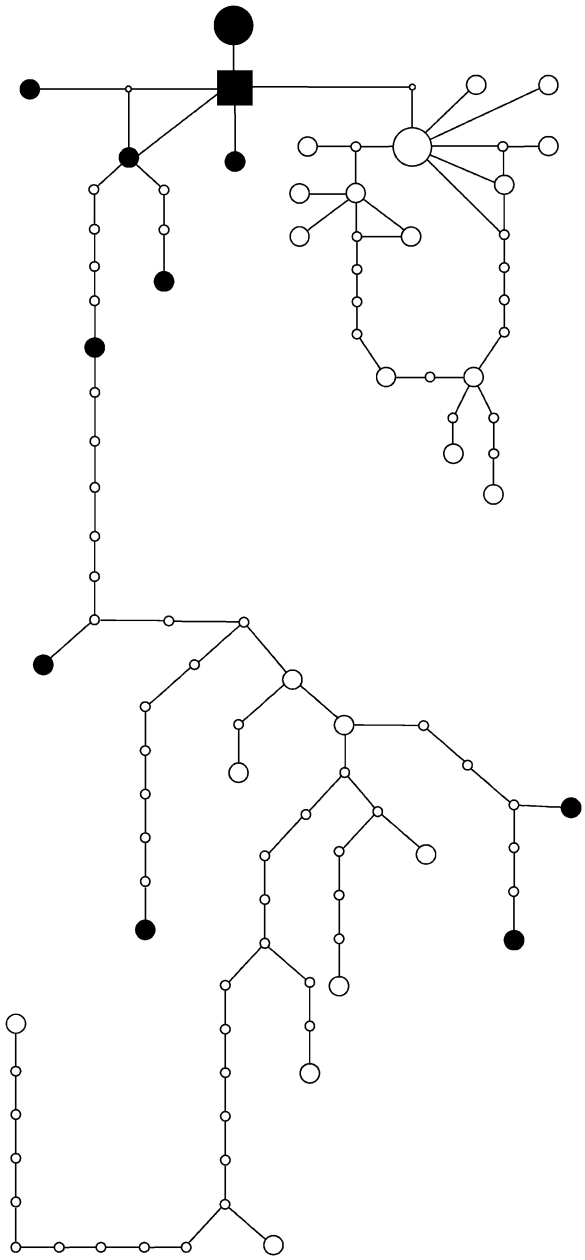

FIG. 4.—Statistical parsimony network of COI haplotypes of *Echinometra lucunter*. Area of each shape is proportional to the number of individuals bearing a haplotype, open shapes indicate haplotypes found in the Caribbean Sea, filled shapes haplotypes found in the Atlantic Ocean. The ancestral haplotype as determined by outgroup weight (Castelloe and Templeton 1994) is depicted as a square, hypothetical haplotypes as small empty circles. Two haplotypes from the Caribbean and two from the Atlantic could not be joined to this network at the 95% confidence limit.

on bindin. The mechanism of selection on bindin of *E. lucunter*, however, probably includes more components than what was demonstrated by Levitan and Farrell. Under the *Strongylocentrotus* model, high sperm density should promote heterozygosity and polymorphism, but bindin of *E. lucunter* has lower variation than that of the other two Neotropical species in this genus. The low variation of *E. lucunter* bindin suggests a role for assortative mating. Assortative mating has been demonstrated by Palumbi (1999) in *Echinometra mathaei*, in which males carrying a particular bindin allele are more likely to fertilize females that carry the same bindin (and the presumably linked bindin receptor) allele. Sperm competition in high sperm densities would favor bindin receptor alleles that are more discriminating and would set both bindin and the bindin receptor in *E. lucunter* on a course of runaway divergence from its sister species that would create a signal of positive selection unrelated to avoidance of hybridization. Whatever the cause of selection on bindin turns out to be, it is certain that this molecule in *E. lucunter* currently shows no pattern of character displacement, and no signature of stronger selection in areas of sympatry relative to areas of allopatry, which suggests that selective forces are likely to operate independently of the challenge of a related species. Sexual selection and sperm competition would be operating throughout the species range, regardless of the presence of a sister species.

Howard (1993) outlined the kinds of evidence needed for the demonstration of reinforcement in nature. The relevant question here is what data would constitute convincing evidence that reinforcement has *not* occurred. A report of negative results, showing that a phenomenon expected to happen actually did not, may be considered as a demonstration that the investigators' imagination in formulating hypotheses was not matched by the potential of the organisms to conform to it. However, reporting the absence of character displacement on a reproductive trait suspected of having evolved under reinforcement is by no means superfluous. Attempts to assess the frequency of reinforcement from analyses of the literature may well suffer from publication bias; it is possible that studies that have encountered evidence of reinforcement are more likely to be published than those that looked for such evidence but failed to find it (Howard 1993; Coyne and Orr 2004; LeGac and Giraud 2008). In the case of the Atlantic species of *Echinometra*, the question was not whether there was speciation by reinforcement, but whether avoidance of hybridization is the selective force that has acted on bindin. That the results of the present study suggest that reinforcement is unlikely as one of these possibilities strengthens the case that intraspecific processes, such as sperm competition, sexual selection, or intersexual conflict, may be more likely explanations for the selection that drives bindin evolution in this species.

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Literature Cited

Akaike H. 1974. A new look at the statistical model identification. IEEE Trans Autom Contr. 19:716–723.

Anisimova M, Bielawski JP, Yang Z. 2001. Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. Mol Biol Evol. 18:1585–1592.

Bermingham EB, Lessios HA. 1993. Rate variation of protein and mtDNA evolution as revealed by sea urchins separated by the Isthmus of Panama. Proc Natl Acad Sci USA. 90:2734–2738.

Biermann CH. 1998. The molecular evolution of sperm bindin in six species of sea urchins (Echinoida: Strongylocentrotidae). Mol Biol Evol. 15:1761–1771.

Butlin RK. 1995. Reinforcement—an idea evolving. Trends Ecol Evol. 10:432–434.

Castelloe J, Templeton AR. 1994. Root probabilities for intraspecific gene trees under neutral coalescent theory. Mol Phylogenet Evol. 3:102–113.

Civetta A, Singh RS. 1995. High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in *Drosophila melanogaster* and *Drosophila virilis* group species. J Mol Evol. 41:1085–1095.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. Mol Ecol. 9: 1657–1659.

Coyne JA, Orr HA. 2004. Speciation. Sunderland (MA): Sinauer.

Darteville E. 1953. Echinides fossliles du Congo et de l'Angola. 2e partie. Desciption systematique des echinides fossiles du Congo et el l'Angola. Ann Mus Roy Congo Belge. 13:1–240.

Dobzhansky T. 1940. Speciation as a stage in evolutionary divergence. Am Nat. 74:312–321.

Excoffier G, Laval L, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online. 1:47–50.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics. 131:479–491.

Franke ES, Babcock RC, Styan CA. 2002. Sexual conflict and polyspermy under sperm-limited conditions: in situ evidence from field simulations with the free-spawning marine echinioid *Evechinus chloroticus*. Am Nat. 160:485–496.

Galindo BE, Vacquier VD, Swanson WJ. 2003. Positive selection in the egg receptor for abalone sperm lysin. Proc Nat Acad Sci USA. 100:4639–4643.

Geyer LB, Palumbi SR. 2003. Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. Evolution. 57:1049–1060.

Geyer LB, Palumbi SR. 2005. Conspecific sperm precedence in two species of tropical sea urchins. Evolution. 59:97–105.

Haygood R. 2004. Sexual conflict and protein polymorphism. Evolution. 58:1414–1423.

Howard DJ. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In: Harrison RG, editor. Hybrid zones and the evolutionary process. New York: Oxford Univversity Press.

Hudson RR. 1987. Estimating the recombination parameter of finite population model without selection. Genet Res. 50: 245–250.

Hudson RR, Kaplan NL. 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics. 111:147–164.

Kamei N, Glabe CG. 2003. The species-specific egg receptor for sea urchin sperm adhesion is EBR1, a novel ADAMTS protein. Genes Dev. 17:2502–2507.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16:111–120.

Kimura M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. Proc Natl Acad Sci USA. 78:454–458.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5:150–163.

Landry C, Geyer LB, Arakaki Y, Uehara T, Palumbi SR. 2003. Recent speciation in the Indo-West Pacific: rapid evolution of gamete recognition and sperm morphology in cryptic species of sea urchin. Proc R Soc Lond B. 270:1839–1847.

LeGac M, Giraud T. 2008. Existence of a pattern of reproductive character displacement in Homobasidiomycota but not in Ascomycota. J Evol Biol. 21:761–772.

Lemmon AR, Smadja C, Kirkpatrick M. 2004. Reproductive character displacement is not the only possible outcome of reinforcement. J Evol Biol. 17:177–183.

Lessios HA. 1979. Use of Panamanian sea urchins to test the molecular clock. Nature. 280:599–601.

Lessios HA. 1981a. Divergence in allopatry: molecular and morphological differentiation between sea urchins separated by the Isthmus of Panama. Evolution. 35:618–634.

Lessios HA. 1981b. Reproductive periodicity of the echinoids *Diadema* and *Echinometra* on the two coasts of Panama. J Exp Mar Biol Ecol. 50:47–61.

Lessios HA. 1991. Presence and absence of monthly reproductive rhythms among eight Caribbean echinoids off the coast of Panama. J Exp Mar Biol Ecol. 153:27–47.

Lessios HA. 2007. Reproductive isolation between species of sea urchins. Bull Mar Sci. 81:191–208.

Lessios HA, Cunningham CW. 1990. Gametic incompatibility between species of the sea urchin *Echinometra* on the two sides of the Isthmus of Panama. Evolution. 44:933–941.

Lessios HA, Kessing BD, Wellington GM, Graybeal A. 1996. Indo-Pacific echinoids in the tropical eastern Pacific. Coral Reefs. 15:133–142.

Levitan DR. 2004. Density-dependant sexual selection in external fertilizers: variances in male and female fertilization success along the continuum from sperm limitation to sexual conflict in the sea urchin *Strongylocentrotus franciscanus*. Am Nat. 164:298–309.

Levitan DR, Ferrell DL. 2006. Selection on gamete recognition proteins depends on sex, density, and genotype frequency. Science. 312:267–269.

Levitan DR, terHorst CP, Fogarty ND. 2007. The risk of polyspermy in three congeneric sea urchins and its implication for gametic incompatibility and reproductive isolation. Evolution. 61:2009–2016.

Li W-H. 1993. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. J Mol Evol. 36:96–99.

McCartney MA, Keller G, Lessios HA. 2000. Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. Mol Ecol. 9:1391–1400.

McCartney MA, Lessios HA. 2002. Quantitative analysis of gametic incompatibility between closely related species of neotropical sea urchins. Biol Bull. 202:166–181.

McCartney MA, Lessios HA. 2004. Adaptive evolution of sperm bindin tracks egg incompatibility in neotropical sea urchins of the genus *Echinometra*. Mol Biol Evol. 21:732–745.

McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. Nature. 351:652–654.

Metz EC, Gomez-Gutierrez G, Vacquier VD. 1998. Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia*. Mol Biol Evol. 15:185–195.

Metz EC, Kane RE, Yanagimachi H, Palumbi SR. 1994. Fertilization between closely related sea urchins is blocked by incompatibilities during sperm-egg attachment and early stages of fusion. Biol Bull. 187:23–34.

Metz EC, Palumbi SR. 1996. Positive selection and sequence rearrangements generate extensive polymorphism in the

gamete recognition protein bindin. Mol Biol Evol. 13: 397–406.
Moore WS. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution. 49:718–726.
Mortensen T. 1943. A monograph of the Echinoidea. Vol. III. Camarodonta II. Copenhagen (Denmark): C.A. Reitzel.
Nei M, Gojobori T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 3:418–426.
Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. New York: Oxford University Press.
Noor MAF. 1999. Reinforcement and other consequences of sympatry. Heredity. 83:503–508.
Palumbi SR. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc Natl Acad Sci USA. 96:12632–12637.
Palumbi SR. 2009. Speciation and the evolution of gamete recognition genes: pattern and process. Heredity. 102:66–76.
Palumbi SR, Cipriano F, Hare MP. 2001. Predicting nuclear gene coalescence from mitochondrial data: the three-times rule. Evolution. 55:859–868.
Palumbi SR, Lessios HA. 2005. Evolutionary animation: how do molecular phylogenies compare to Mayr's reconstruction of speciation patterns in the sea? Proc Natl Acad Sci USA. 102(1 Suppl):6566–6572.
Pamilo P, Bianchi NO. 1993. Evolution of the Zfx and Zfy genes: rates and interdependence between the genes. Mol Biol Evol. 10:271–281.
Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics. 14:817–818.
Rice WR. 1989. Analyzing tables of statistical tests. Evolution. 43:223–225.
Riginos C, McDonald JH. 2003. Positive selection on an acrosomal sperm protein, M7 lysin, in three species of the mussel genus *Mytilus*. Mol Biol Evol. 20:200–207.
Riginos C, Wang D, Abrams AJ. 2006. Geographic variation and positive selection on M7 lysin, an acrosomal sperm protein in mussels (*Mytilus* spp.) Mol Biol Evol. 23:1952–1965.
Rozas J, Rozas R. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics. 15:174–175.
Seutin G, White BN, Boag PT. 1991. Preservation of avian blood and tissue samples for DNA analyses. Can J Zool. 69:82–90.
Servedio MR. 2004. The what and why of research on reinforcement. PLoS Biol. 2:2032–2035.
Swanson WJ, Vacquier VD. 2002a. The rapid evolution of reproductive proteins. Nat Rev Genet. 3:137–144.
Swanson WJ, Vacquier VD. 2002b. Reproductive protein evolution. Annu Rev Ecol Syst. 33:161–179.
Swanson WJ, Wong A, Wolfner MF, Aquadro CF. 2004. Evolutionary expressed sequence tag analysis of Drosophila female reproductive tracts identifies genes subjected to positive selection. Genetics. 168:1457–1465.
Swofford DL. 2001. PAUP*: phylogenetic analysis using parsimony *and other methods, Version 4. Sunderland (MA): Sinauer.
Templeton AR. 1981. Mechanisms of speciation—a population genetic approach. Annu Rev Ecol Syst. 12:23–48.
Templeton AR, Crandall KA, Sing CF. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data III. Cladogram estimation. Genetics. 132:619–633.
Vacquier VD, Moy GW. 1977. Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. Proc Natl Acad Sci USA. 74:2456–2460.
Wong WSW, Yang Z, Goldman N, Nielsen R. 2004. Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identifying positively selected sites. Genetics. 168:1041–1051.
Yang Z. 1997. PAML, a program package for phylogenetic analysis by maximum likelihood. CABIOS. 13:555–556.
Yang Z, Nielsen R. 2002. Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol Biol Evol. 19:908–917.
Yang Z, Nielsen R, Goldman N, Pedersen A-MK. 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics. 155:431–449.
Yang Z, Wong WSW, Nielsen R. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. Mol Biol Evol. 22:1007–1118.
Yang ZH. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol. 15:568–573.
Zhang JZ, Nielsen R, Yang ZH. 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol Biol Evol. 22:2472–2479.
Zigler KS, Lessios HA. 2003. Evolution of bindin in the pantropical sea urchin *Tripneustes*: comparisons to bindin of other genera. Mol Biol Evol. 20:220–231.
Zigler KS, Lessios HA. 2004. Speciation on the coasts of the new world: phylogeography and the evolution of bindin in the sea urchin genus *Lytechinus*. Evolution. 58:1225–1241.
Zigler KS, McCartney MA, Levitan DR, Lessios HA. 2005. Sea urchin bindin divergence predicts gamete compatibility. Evolution. 59:2399–2404.
Zigler KS, Raff EC, Popodi E, Raff RA, Lessios HA. 2003. Adaptive evolution of bindin in the genus *Heliocidaris* is correlated with the shift to direct development. Evolution. 57:2293–2302.
Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [PhD dissertation]. [Austin (TX)]: The University of Texas at Austin.

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