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TESTS OF PHYLOGEOGRAPHIC MODELS WITH NUCLEAR AND MITOCHONDRIAL DNA SEQUENCE VARIATION IN THE STONE CRABS, *MENIPPE ADINA* AND *MENIPPE MERCENARIA*

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Abstract.—Evolutionary relationships among stone crabs (*Menippe*) from the Gulf of Mexico and western Atlantic were investigated by comparisons of restriction sites within anonymous nuclear DNA sequences and nucleotide sequences of both mitochondrial and a duplicated nuclear form of the mitochondrial large subunit ribosomal RNA (LSrDNA) gene. A survey of over 100 restriction sites by Southern blot analysis with 10 anonymous nuclear DNA sequence probes failed to reveal any differences between *Menippe adina* and *M. mercenaria*. Sequence comparisons of both mitochondrial and nuclear forms of the LSrDNA gene also did not distinguish these species. Although both LSrDNA gene sequences were variable, some haplotypes were shared by the two species, implying either incomplete gene lineage sorting or introgressive hybridization. Based on molecular clock calibrations, we estimate that all of the observed mitochondrial LSrDNA sequences share a common ancestor between 1.5 and 2.7 million years before present (M.Y.B.P.). However, because identical sequences are shared by the two species, these data are also compatible with a more recent common ancestry. These findings conflict with a previously proposed biogeographic scenario for North American *Menippe*, which featured a relict hybrid zone on the Atlantic Coast. We suggest an alternative scenario based on relatively recent events and ongoing, rather than historical, gene flow.

Key words.—Hybrid zone, marine biogeography, *Menippe*, mitochondrial DNA, phylogeography, Suwanee Straits.

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The North American distribution of stone crabs (genus *Menippe*) is typical of many low intertidal and shallow subtidal invertebrates. One form is found in the western Gulf of Mexico, while a second form, a sister taxon, ranges from peninsular Florida to North Carolina on the Atlantic coast. These two forms were initially considered to be a single species, *Menippe mercenaria* (Say 1818). Later, on the basis of allozyme and coloration differences, Bert (1986) suggested that they should be considered semispecies. Williams and Felder (1986) determined that the western Gulf of Mexico form was sufficiently distinct by morphological criteria to be designated a separate species, which they named *Menippe adina*. The two species intergrade along the western coast of Florida, in an area that is considered to be a hybrid zone (Bert 1986; Williams and Felder 1986; Bert and Harrison 1988; Bert et al. 1996). A third species, *M. nodifrons*, is restricted to reef habitats primarily in the Caribbean and is quite distinct from either *M. mercenaria* or *M. adina*.

A number of biogeographic scenarios could account for the separation of western Gulf from Atlantic populations of *Menippe*. Plausible mechanisms can be found in glacial episodes that controlled the emergence of landmasses and influenced the direction of prevailing oceanic currents (e.g., Brunner 1982; Cronin 1988). However, Bert (1986) reported a second hybrid zone (Fig. 1), within the range of *M. mercenaria*, which suggested a more complex interpretation. On the basis of the location of this putative Atlantic hybrid zone, she proposed that gene flow from the Gulf of Mexico occurred in the remote past, across the Florida peninsula through the Okeefenokee Trough. There is geological evidence that high sea levels created a waterway, the Suwanee Straits, through which a current flowed from the Gulf of Mexico to the Atlantic (Riggs 1984). This scenario requires that the characteristics that differentiate *M. adina* from *M. mercenaria* were developed before the last opening of the Suwanee Straits, which is unlikely to have been more recent then the late Pliocene (Olsen 1968), and perhaps as remote as the middle Miocene (Olsen 1968; Brooks 1973). In Bert's (1986) proposed evolutionary history for *Menippe*, this differentiation began during the terminal Miocene glaciation, four to five M.Y.B.P. The occurrence and timing of the events in the Atlantic hybrid zone scenario for *Menippe* have important implications for the biogeographic interpretation of this region as a whole.

Without direct evidence of past contact between species, the identification of relict hybrid zones can be ambiguous. As noted by Bert (1986), it is difficult to distinguish the effects of secondary contact and hybridization from a background of intraspecific variation when the presumed events occurred in the past. The morphometric study of Williams and Felder (1986), which used a discriminant analysis of carapace shape and cheliped characters, identified individuals with intermediate morphologies in the western Florida hybrid zone, but found no evidence of hybridization in the Atlantic. Furthermore, allozyme alleles that were used to identify hybrids because they were considered diagnostic for *M. adina* (Bert 1986; Bert and Harrison 1988) were actually present elsewhere in the range of *M. mercenaria*, although at low frequencies (fig. 4 in Bert and Harrison 1988). Thus more definitive evidence for a relict hybrid zone on the Atlantic coast is needed.

We examined nuclear DNA sequence polymorphisms and mitochondrial large subunit ribosomal DNA (LSrDNA) sequence variants in an effort to find diagnostic markers that would consistently differentiate *M. adina* from *M. mercenaria*, and thus provide definitive criteria for the identification

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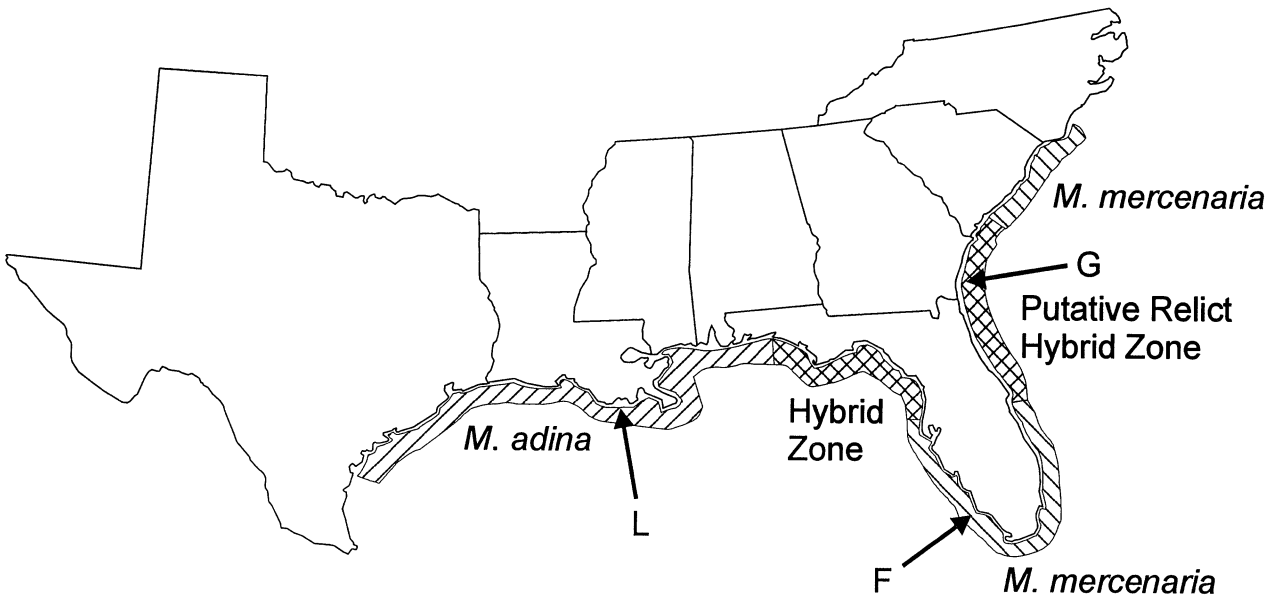

FIG. 1. Collecting sites and reported distributions of *Menippe adina, M. mercenaria*, and hybrids. L, Terrebonne Bay, Louisiana; F, Rookery Bay, Florida; G, Saint Simon Sound, Georgia.

of hybrids. Because the Atlantic hybrid zone hypothesis implies a relatively long period of separation between the species, we expected to find detectable divergence in both mitochondrial and nuclear DNA sequences. Furthermore, we had hoped that a phylogeographic analysis of the relationships of mtDNA lineages and their geographic distributions would help to clarify the origins of the putative Atlantic hybrid zone. Instead, we found a lack of interspecific divergence in anonymous nuclear sequences, the mitochondrial LSrDNA sequence, and a nuclear copy of the mitochondrial LSrDNA sequence. These findings imply a relatively recent common ancestry for the two species and suggest a revised explanation for the appearance of an Atlantic hybrid zone.

MATERIALS AND METHODS

Stone crabs (genus *Menippe*) were collected in wire crab pots by local fishermen and by DLF from two localities that have been considered pure populations of each species, and a third locality within the putative Atlantic hybrid zone (Bert 1986; Williams and Felder 1986; Bert and Harrison 1988). The locality for *M. adina* was upper Terrebonne Bay near Cocoderie, Louisiana; for *M. mercenaria*, Rookery Bay, north of Marco Island near Naples, Florida; and for putative hybrids, Saint Simon Sound near Brunswick, Georgia (Fig. 1).

Whole genomic DNA was isolated from testes by the procedure described in Neigel et al., (1991). Within 24 hours of arrival, male crabs were placed on ice until cessation of movement. Testicular tissue was removed by dissection, ground under liquid nitrogen, transferred to 1.2 volume of lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, and 0.1 mg/ml Proteinase K), and incubated overnight with gentle shaking at 50°C. The resulting lysates were either purified directly by cesium gradient centrifugation or first extracted with a 25:24:1 mixture of buffered phenol, chloroform, and isoamyl alcohol. The ethidium bromide was removed by extraction with CsCl saturated isopropanol. The DNA was then dialyzed against several changes of dialysis buffer (10 mM Tris pH 8.0, 10 mM NaCl, 0.1 mM EDTA pH 8.0; Palmer 1986). Individuals and genomic DNA samples were sequentially numbered as they were collected.

A small genomic library was made from partial *Sau*3AI digests of *Menippe* genomic DNA. The digests were electrophoresed on 0.7% ethidium-stained agarose gels, and DNA was electroeluted from the portion of the gel containing fragments in the range of 2–5 kb. These size-selected fragments were then cloned into the *Bam*HI site of the plasmid vector pUC 19. Clones containing repetitive sequences were identified by the "phi-screen" method (Wichman et al. 1985; Neigel et al. 1991), and excluded from further analysis. The remaining clones, which were considered anonymous single-copy or low-copy number sequences, were used as hybridization probes. Southern blot analysis of total genomic DNA probed with cloned *Menippe* mitochondrial genome sequences indicated that the mitochondrial genome is a minor component of our genomic DNA preparations (data not shown). Thus we expect most, if not all, of the cloned sequences to have originated in the nuclear genome.

Hybridization probes obtained from the procedure described above were used to survey restriction fragments in *M. mercenaria* and *M. adina*. Samples of genomic DNA (15–20 μg) from four individuals of each species were individually digested with restriction endonucleases that recognize either four or six base sequences, electrophoresed in 1.5% and 1.0% agarose gels, respectively, and blotted onto Magnagraph nylon (Micron Separations, Inc.) membranes. Ten hybridization probes were used in combination with some of the following nine restriction endonucleases: *Eco*RI, *Hae*III, *Hha*I, *Hin*dIII, *Msp*I, *Pst*I, *Rsa*I, *Xba*I, and *Xho*I. A total of 31 combinations of probe and enzyme were used. Labeling

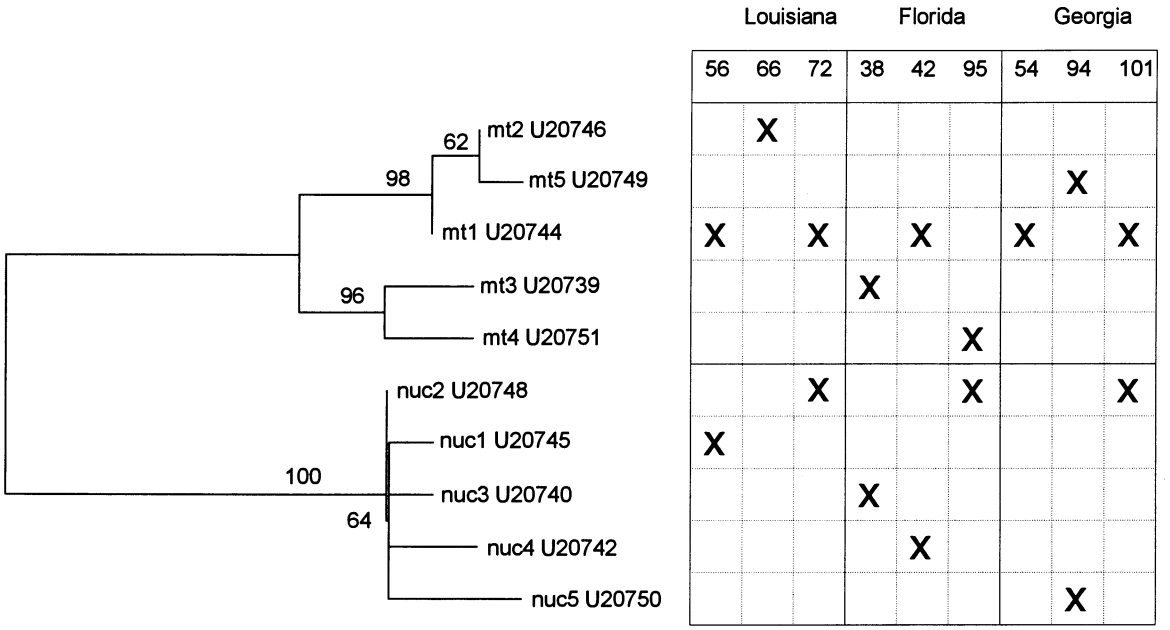

FIG. 2. Neighbor-joining phenogram of LSrDNA sequences and the occurrence of these sequences in individual *Menippe* specimens. Mitochondrial sequences are labeled as mtl to mt5, nuclear sequences as nucl to nuc5; GenBank accession numbers are shown in parentheses. The phenogram was generated by neighbor-joining of Kimura two-parameter distances. Numbers at nodes are percentages of 1000 bootstraps replicates. The rows of the table are aligned with the phenogram to show the distribution of the sequences among individuals. Each column corresponds to a distinct individual; the number at the top of each column corresponds to a specimen number that was assigned when the individual was collected. Individuals are grouped by species and location.

of probes with digoxigenin, hybridization, and immunological detection of probes were performed according to the manufacturer's (Boehringer Mannheim) specifications for Genius nonradioactive detection methods. Only bands that were clearly distinct were scored. Restriction fragment sizes were estimated to within ten base pairs (bp) from their observed mobility using the algorithm of Schaffer and Sederoff (1981).

A 565-bp portion of the mitochondrial large subunit ribosomal DNA (LSrDNA) and a 567- bp translocated nuclear copy of this region were amplified and sequenced as described elsewhere (Schneider-Broussard and Neigel 1997). Mitochondrial sequences were obtained from three individuals at each of the three collecting localities. Nuclear sequences were obtained from seven of these nine individuals: all three from the *M. adina* locality and two each from the *M. mercenaria* locality and the locality within the putative hybrid zone. All sequences have been deposited in GenBank, and accession numbers are shown in Figure 2. The program MEGA (Kumar et al. 1993) was used to calculate Kimura two-parameter sequence distances and their standard errors (Kimura 1980), construct a phenogram based on a neighbor-joining algorithm (Saitou and Nei 1987), perform a bootstrap test of the neighbor-joining phenogram with 1000 replicates, and construct a cladogram based on maximum parsimony criteria. Divergence times between mitochondrial LSrDNA sequences were estimated utilizing published molecular clock calibrations for other Brachyura (Sturmbauer et al. 1996; Schubart, et al. 1998). Two methods were used to construct confidence limits for estimates of proportions of nucleotide and restriction site differences between sequences. For comparisons between sequences that were not identical, 95% confidence limits were approximated as plus or minus two standard errors of the Kimura two-parameter sequence distance. For identical sequences, Kimura's (1980) estimator for the standard error of this distance is zero. Therefore for identical sequences, as well as for proportions of restriction sites, we used the confidence limits for proportions listed in table 23 of Rohlf and Sokal (1981).

RESULTS

Anonymous Low Copy Sequence Restriction Site Survey

The survey of restriction sites detected by hybridization with anonymous single- or low-copy genomic DNA probes revealed only one definite polymorphism and no diagnostic differences between *M. adina* and *M. mercenaria*. The numbers of restriction fragments observed from combinations of hybridization probe and restriction endonuclease are reported in Tables 1 and 2. A minimum of 54 six-base recognition sites and 50 four-base restriction sites were surveyed, representing a minimum total of 524 bases. For eight of the probes it could be concluded that some of the hybridizing fragments were not contiguous within the genome because the probe was not of sufficient length to span them. Therefore these probes must have hybridized to multiple, noncontiguous sequences. This indicates that some of the clones selected by the phi-screen procedure hybridize to more than one genomic sequence.

The single probe that revealed definite polymorphisms (pMACC23) did so in combination with any of several restriction endonucleases. Because relative differences in frag-

TABLE 1. Southern hybridization analysis of six base restriction sites. Hybridizing fragments are listed for each combination of genomic probe and six base restriction endonuclease. The maximum number of contiguous fragments and the minimum and maximum numbers of restriction sites surveyed are based on the probe and hybridizing fragment lengths. Probe and fragment lengths are given in kbp.

Probe	Probe length	Restriction endonuclease	Fragment lengths	Maximum contiguous	Minimum sites	Maximum sites
5	2.1	*Pst*I	0.88	1	2	2
6	0.90	*Hin*dIII	0.58	1	2	2
6	0.90	*Pst*I	1.6, 1.2, 0.90, 0.68	3	6	8
6	0.90	*Xho*I	1.7	1	2	2
23	1.2	*Hin*dIII	0.56	1	2	2
23	1.2	*Xho*I	1.7	2	2	2
25	0.54	*Pst*I	1.6, 0.80, 0.62	2	5	6
30	1.7	*Eco*RI	1.6	1	2	2
30	1.7	*Xho*I	1.6	1	2	2
33	3.7	*Pst*I	1.9, 1.1, 1.0, 0.91, 0.54	5	6	10
36	2.0	*Eco*RI	2.2, 1.6	2	3	4
36	2.0	*Pst*I	0.63	1	2	2
55	0.37	*Hin*dIII	1.6, 1.1, 0.60	2	5	6
55	0.37	*Pst*I	1.6, 0.64	2	3	4
55	0.37	*Xba*I	1.7, 1.0, 0.59	2	5	6
55	0.37	*Xho*I	1.6, 0.63	2	3	4
58	0.44	*Pst*I	1.7	1	2	2
				Totals	54	66

ment lengths were similar for different enzymes (Table 3), it appears that these polymorphisms represent variation in sequence length rather than variation in the presence of restriction sites. This is also suggested by the sequence of pMACC23, which contains a series of dinucleotide repeats (Fig. 3). Such repeats are characteristic of "microsatellite" sequences, which are often extremely polymorphic in the number of repeats, and thus sequence length (Tautz 1989). Distinct allele combinations were associated with each individual, but there were no consistent differences between species (Table 3).

The original intention of the genomic restriction fragment survey was to identify diagnostic markers for *M. mercenaria* and *M. adina*. Instead, it demonstrated the similarity of the two species. Using the most conservative estimate of the number of bases surveyed (524) the observed value of zero base substitutions corresponds to a 95% confidence limit of 0.0% to 0.57% sequence divergence. This range is significantly lower then would be expected from estimates of sequence divergence rates for other taxa and the time span implied by the Atlantic hybrid zone scenario. For example, Vawter and Brown (1986) estimated an overall sequence divergence rate of 0.3% to 3% per million years for single copy nuclear DNA sequences in sea urchins and primates. Using a conservatively low value (0.3%) for the rate of sequence divergence, and 3.5 million years as the minimum time of separation implied by the Atlantic hybrid zone scenario, we would expect at least 1% sequence divergence between *M. mercenaria* and *M. adina*. Thus even a conservative calculation of the sequence divergence expected under the Atlantic hybrid zone scenario is above the upper confidence limit for our estimate based on nuclear restriction sites.

Analysis of LSrDNA Sequences

Both neighbor-joining (NJ) and maximum-parsimony (MP) methods distinguished mitochondrial from nuclear forms of the LSrDNA sequence (Fig. 2). Among the nine mitochondrial sequences sampled, there were five distinct haplotypes (designated mt1 through mt5) with 11 polymorphic positions. Six of the polymorphic positions were phylogenetically informative. Both NJ and MP methods divided the mitochondrial haplotypes into two groups, however, these groups do not correspond to the two species of *Menippe* (Fig. 2). One group, represented in 96% of the NJ bootstrap replicates, consisted of two haplotypes (mt3 and mt4) that were found in *M. mercenaria* from the Florida locality. The other group, represented in 98% of the bootstrap replicates, included haplotypes from all three localities, which represent both species and the putative Atlantic hybrid zone within the range of *M. mercenaria*. This group included the most common mitochondrial LSrDNA haplotype (mt1), which itself was found in individuals from all three localities.

Among the seven nuclear LSrDNA sequences there were five distinct haplotypes (designated nuc1 through nuc5) with seven polymorphic positions. Neighbor-joining analysis failed to resolve any major clusters among these sequences; they form a single group with little divergence between them (Fig. 2). Maximum parsimony analysis could not resolve subgroups of nuclear haplotypes because none of the polymorphic positions were phylogenetically informative at that level. One common haplotype (nuc2) was found in individuals from each of the three collection localities, the other haplotypes were unique to single individuals.

Estimated Divergence Times between Mitochondrial LSrDNA Sequences

We used published estimates of LSrDNA divergence rates in other brachyuran crab lineages to estimate divergence times between *Menippe* mitochondrial LSrDNA sequences. For fiddler crabs lineages separated by the rise of the Isthmus of Panama, Sturmbauer et al. (1996) estimated a rate of 0.9% sequence divergence per million years. There are also two estimates for trans-isthmus species pairs within the genus *Sesarma*: 0.65% per million years for the *reticulatum* group

TABLE 2. Southern hybridization analysis of four base restriction sites. Hybridizing fragments are listed for each combination of genomic probe and four base restriction endonuclease. The maximum number of contiguous fragments and the minimum and maximum numbers of restriction sites surveyed are based on the probe and hybridizing fragment lengths. Probe and fragment lengths are given in kbp.

Probe	Probe length	Restriction endonuclease	Fragment lengths	Maximum contiguous	Minimum sites	Maximum sites
5	2.1	*Hae*III	0.48	1	2	2
5	2.1	*Msp*I	0.77	1	2	2
5	2.1	*Rsa*I	0.57, 0.37	2	3	4
6	0.90	*Msp*I	1.9, 1.7, 1.0, 0.87, 0.71, 0.66, 0.57	3	12	14
6	0.90	*Rsa*I	0.51, 0.45, 0.39, 0.34, 0.28	4	7	10
22	0.54	*Msp*I	1.0	1	2	2
22	0.54	*Rsa*I	0.75, 0.50	2	3	4
23	1.2	*Hae*III	0.58, 0.53	2	3	4
23	1.2	*Msp*I	0.60	1	2	2
33	3.8	*Msp*I	1.1	1	2	2
55	0.37	*Hae*III	0.26	1	2	2
55	0.37	*Hha*I	1.0, 0.84, 0.60	2	5	6
58	0.44	*Msp*I	1.0, 0.86	2	3	4
58	0.44	*Rsa*I	0.57	1	2	2
				Totals	50	60

and 0.88% per million years for the *sulcatum* group (Schubart et al. 1998). Based on these rates, estimated divergence times between *Menippe* mitochondrial LSrDNA sequences range from zero for identical sequences to between 1.5 and 2.7 million years for the most divergent sequences. Divergence times corresponding to the upper and lower confidence limits for these estimates range from 0.36 to 4.5 million years. For identical sequences, the 95% confidence interval for the percentage of nucleotide differences is 0.0–0.6%. Using the above molecular clock calibrations, the upper limit of this interval corresponds to 0.7 to 0.9 million years.

DISCUSSION

This study was initially undertaken to develop genetic markers for *M. mercenaria* and *M. adina* that could be used to identify hybrids. We failed to find such markers for two reasons. First, there was a general lack of variation for restriction sites in anonymous single- or low- copy sequences. Second, for the variation that we did find in one microsatellite sequence and in mitochondrial and nuclear LSrDNA sequences, there were no consistent differences between species. Although each locality was represented by a small number of individuals (three) and each taxon was represented by only one locality, we can conclude that none of the polymorphisms that we detected were "diagnostic" for either species. This was not surprising for the microsatellite sequence. Frequent length mutations in microsatellite sequences would prevent the fixation of alleles within a species and would also lead to independent generation of the same alleles in different species. In contrast, gene genealogies that can be inferred from sequences that evolve by point mutations would be expected to eventually reach reciprocal monophyly after speciation (Neigel and Avise 1986). Distinct sequences, or sets of related sequences, would therefore characterize species that have been separate for a sufficient period of time. Reciprocal monophyly has not been reached for LSrDNA sequences from the two *Menippe* species. For both mitochondrial and nuclear forms of the sequence, there was a haplotype that occurred at all three localities. Thus larger samples may have revealed haplotype frequency differences between species, but not diagnostic sequences.

TABLE 3. Polymorphic restriction fragments hybridizing with probe 23. Each individual had one or two hybridizing bands in the size range characteristics of the polymorphism. Sizes are in bp, estimated to within 10 bp.

	*Hae*III		*Hha*I	
Individual	Band 1	Band 2	Band 1	Band 2
M. adina				
1	990	1050	1260	1320
2	1100	1150	1350	1420
3	1150		1420	
4	1050		1320	
M. mercenaria				
5	990	1100	1260	1350
6	990		1260	
7	1050	1100	1320	1350
8	1050	1150	1320	1420

Both NJ and MP analyses identified two major groups of mitochondrial LSrDNA haplotypes. These groups are strongly supported in bootstrap replicates of the NJ algorithm. The distribution of these two groups of haplotypes between the two *Menippe* species is paraphyletic (Fig. 2). Although one group, which consisted of haplotypes mt3 and mt4, was restricted to *M. mercenaria*, the other group was represented in both species and included one haplotype (mt1) found at all three localities. With additional sampling, we may have found a polyphyletic distribution of haplotypes, with both groups of haplotypes represented in both species. However, we can exclude the possibility that further sampling would yield monophyletic distributions of haplotypes. Paraphyletic and polyphyletic distributions of mitochondrial lineages can be explained in two ways (Neigel and Avise 1986). First, it is possible for haplotypes that were once unique to a single species to appear in other species as the result of introgressive hybridization. In this case, the atypical *M. mercenaria* sequence may have originated within a *M. adina* population, and appeared in the Naples, Florida, population as the result of introgressive hybridization. In support of this interpreta-

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GATCAGCGAA GTTAAGCAAC GTCGGGTCTG GATAGTACTT GGATGGGTGA 50
CCGCCTGGGA ACACCAGATG TTGTTGGCAT CCAATTATTT TTTTTTTTTT 100
CTTTCTTTCT TCCTTCCTTC CTTTCTTTTT TTTCATTCAT TCTCTCTCTC 150
TCTCTCTCTC TCTCTCTCTC TCCAATTCTT CTTCCTCCCA TTTTCAATTC 200
CATTTTTATA CCAGTTTCCC CTAAGAACCC CGCCCCCCTN CTTCCCCCCT 250
ATATATATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT 300
ATTTTTTTTT TGGCCCTTTT CTTGCCGGAA CACTTCTTAT ATGAACGCAT 350
AATTAATGTG GCTAGCTTAT CTTCAACACA CCACCAAGCC AG
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FIG. 3. Sequence of the portion of probe 23 that hybridizes to polymorphic fragments. Dinucleotide repeats are underlined.

tion, Bert and Harrison (1988) reported that the hybrid zones contained a large proportion of *M. mercenaria* phenotypes with *M. adina* genotypes. In addition, Bert (per. comm.) has suggested that the hybrid zone in the northeastern Gulf of Mexico is broader than originally reported and may include the Naples, Florida, population that we considered pure *M. mercenaria*. The other possibility is that these sequences represent mitochondrial lineages that predate the separation of the two species and their present distribution reflects incomplete lineage sorting. The occurrence of multiple mitochondrial lineages within a species is the norm, and the estimated divergence time between these sequences (1.5 to 2.7 million yr) is within the range observed for other marine invertebrate species (e.g., Reeb and Avise 1990). Furthermore, the lack of divergence in nuclear encoded LSrDNA sequences between the two species and the lack of restriction site differences in anonymous low-copy nuclear sequences imply that these species have a very recent common ancestor. This condition is associated with a high probability of paraphyly in mitochondrial lineages (Neigel and Avise 1986). Thus the evidence at hand does not allow us to reject either of these interpretations.

We used molecular evolutionary rates calibrated for other brachyuran crustaceans to estimate divergence times between *Menippe* LSrDNA sequences. There are several caveats that apply to such estimates (Hillis et al. 1996). Variance in estimates of divergence time is expected from sampling a finite number of bases and possibly actual fluctuations in underlying evolutionary rates. Thus we should not expect precise matches between our estimates and the dates of geological events that may have been important in the separation of lineages leading to *M. adina* and *M. mercenaria*. We have attempted to quantify some of this uncertainly by using confidence limits for estimates of sequence divergence and by considering all available brachyuran LSrDNA sequence molecular clock calibrations. Our estimate of 1.5 to 2.7 million years divergence is considerably higher than Bert et al.'s (1996) estimate of 0.8 to 0.9 million years for the most dissimilar mtDNA haplotypes found in their survey of the northwest Florida hybrid zone. However, the latter estimate is based on 43 restriction sites that represent only 258 nucleotide bases and a molecular clock calibration for a caridean (snapping shrimp) lineage rather than a brachyuran lineage.

Our estimate of the time at which *M. adina* and *M. mercenaria* began to diverge depends on how the paraphyletic distribution of mtDNA lineages is interpreted (Fig. 4). If we assume that the occurrence of identical sequences in *M. mercenaria* and *M. adina* represents introgression, then our estimate should be based on the comparison between the dissimilar *M. adina* and *M. mercenaria* sequences (Fig. 4, A vs. C), which corresponds to estimates of divergence time between 1.5 and 2.7 million years, with an upper confidence limit of 4.5 million years. These estimates place the initial separation of *M. adina* from *M. mercenaria* after the Miocene opening of the Suwanee Straits, and not much before a possible second opening in the late Pliocene. In contrast, the incomplete lineage sorting interpretation implies that the largest observed differences between sequences represent ancestral polymorphisms dating from a time prior to the separation of *M. adina* from *M. mercenaria* (Fig. 4, D vs. F). In this case, the appropriate sequence comparison for estimating divergence time between species would be between the most similar haplotypes (Fig. 4, D vs. E). By this interpretation, our estimate of divergence time would be zero. Even with the slowest molecular clock rate calibration for Brachyura, the upper confidence limit of this estimate corresponds to a time (0.9 M.Y.B.P.) that excludes both Miocene and Pliocene events.

It is difficult to reconcile Bert's scenario of a relict hybrid zone in the Atlantic with the occurrence of identical mitochondrial LSrDNA sequences in both *M. adina* and the putative hybrid zone population. If the Atlantic hybrid zone is a relic of an event that ended at least several million years ago, then at least several million years of subsequent divergence should separate sequences that are presently in populations of *M. adina* from those that had introgressed into Atlantic *M. mercenaria* (Fig. 4, A vs. B). Because no divergence is observed and identical sequences are unlikely to have been separated for more than 0.9 million years, it would appear that a much more recent connection exists between western Gulf and Atlantic populations of *Menippe*.

The confidence interval for our estimate of divergence between the most dissimilar LSrDNA haplotypes does not exclude a separation that may have followed the rise of the Isthmus of Panama, approximately 3 M.Y.B.P. (Savin and Douglas 1985). This event produced profound changes in circulation within the Gulf of Mexico (Riggs 1984) that may have isolated Gulf populations from Atlantic populations. Stanley's (1986) paleontological investigation of bivalves led him to hypothesize that with the closing of circulation from the Pacific Ocean, the altered course of the Gulf Stream produced upwelling across the mouth of the Gulf, which served as a barrier to gene flow. Glacial-associated climate changes in the Gulf of Mexico may have selected for adaptations to lower temperatures and decreased salinity during periods of isolation (Bert 1986). Brown and Bert (1993) have shown that *M. adina* larvae are more cold tolerant than are *M. mercenaria* larvae. There are also differences in sub-

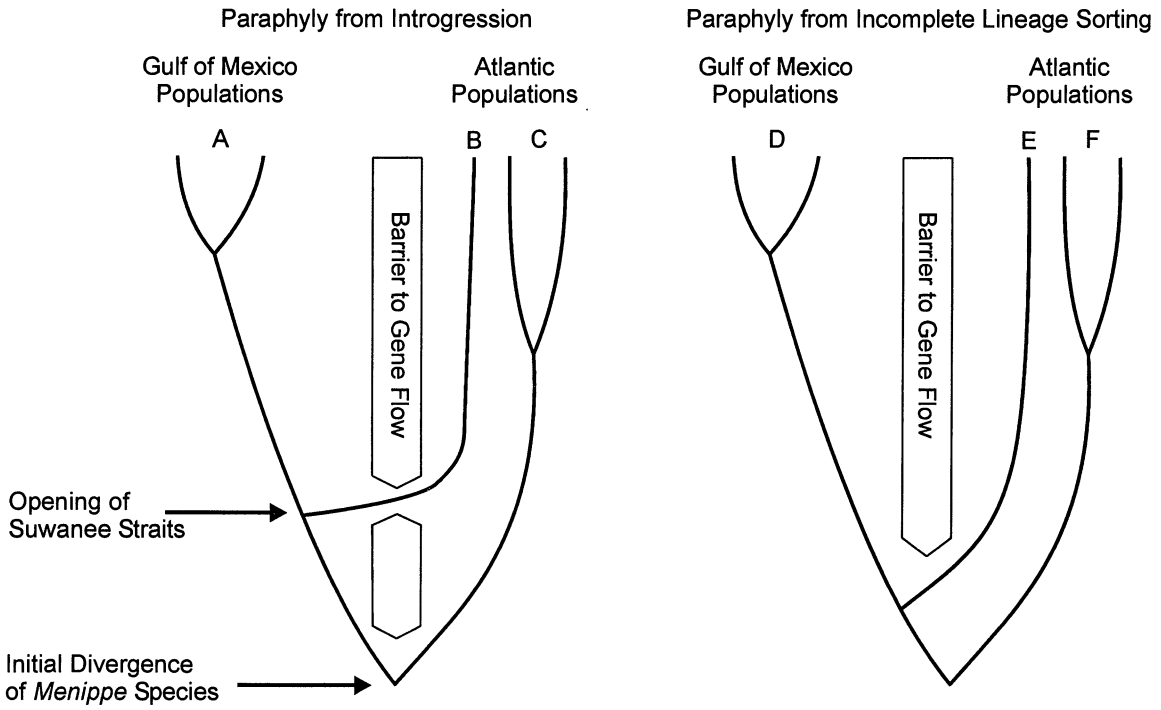

FIG. 4. Alternative biogeographic scenarios for gene flow from *Menippe adina* into Atlantic populations of *M. mercenaria*.

strate preference, which may now act to maintain the distributions of *M. adina* and *M. mercenaria*. Adults of *M. adina* are found to prefer oyster beds, whereas *M. mercenaria* prefers seagrass beds and emergent rocks (Bert 1986; Williams and Felder 1986; Stuck and Perry 1992). Substrate preferences have been shown to have caused a break in the distributions of fiddler and horseshoe crab in Apalachee Bay, Florida, where the dominant substrate type changes from the terrigenous sediments of the western Gulf of Mexico to carbonate sediments around the coast of Florida (Barnwell and Thurman 1984).

Our estimate of divergence time between the most similar (identical) LSrDNA haplotypes shared by *M. adina* and *M. mercenaria* is zero. Thus our data are also consistent with scenarios involving relatively recent climate changes that occurred during the Pleistocene. Latitudinal displacements in the ranges of Gulf of Mexico biota that are likely to have accompanied Pleistocene glaciations may have generated or augmented isolation and adaptive divergence of trans-Floridian species pairs such as *M. adina* and *M. mercenaria* (Felder and Staton 1994).

It is unlikely that adaptation to different habitats would maintain a complete barrier to gene flow between Gulf of Mexico and Atlantic populations of *Menippe*. Episodic gene flow between populations of *M. adina* in the Gulf of Mexico and populations of *M. mercenaria* as far removed as the coast of Georgia could be effected by the occasional loop current (Maul 1976; Hovis et al. 1986) that enters the Gulf of Mexico. Maul (1977) has shown that this current enters the Gulf of Mexico annually and extends to the tip of the Mississippi Delta. The development of a current between April and September, and especially in May, when large numbers of *Menippe* zoeae are in the water column (Stuck and Perry 1992), could transport *M. adina* larvae as far as Georgia. As in the western Gulf of Mexico, the physical environment of the coasts of Georgia and South Carolina are characterized by river outflow and sediment. Larvae of Gulf-adapted *M. adina* may thus be favored in these environments as well.

Regardless of which scenario is used to explain the initial isolation and divergence of *M. adina* and *M. mercenaria*, at least some aspects of the distribution of genetic markers in the Gulf of Mexico and Atlantic must be explained in terms of relatively recent processes or events. Because *Menippe* larvae are capable of long-distance dispersal, it is necessary to explain how these distributions have been maintained despite a high potential for gene flow. Some hybrid zones appear to have been maintained for up to thousands of years as "tension zones," in which dispersal into the tension zone from both parental species is balanced by selection against hybrids (Barton and Hewitt 1985). However, because the putative Atlantic hybrid zone between *M. mercenaria* and *M. adina* is isolated from *M. adina*, selection favoring hybrids would be required to explain the hybrid zone's long-term persistence. A more parsimonious explanation for the appearance of a subset of "*adina*-like" markers along the Atlantic coast is that positive selection has both established and maintained variation in this region. It may therefore be unwarranted to look for evidence of remote historical events in a geographic pattern that has likely been reshaped by more recent effects of environmental selection and gene flow.

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