Cytonuclear Patterns of Genetic Diversity and the Intricate Evolutionary History of the Inland Silverside (*Menidia beryllina*)

KENNETH J. OSWALD, JAMES M. GRADY, AND JOSEPH M. QUATTRO

From the Department of Biological Sciences, University of South Carolina, Columbia, SC 29208 (Oswald and Quattro); and the Department of Biological Sciences, University of New Orleans, New Orleans, LA 70148 (Grady). Kenneth J. Oswald is now at the Molecular Ecology Research Branch, US Environmental Protection Agency, Cincinnati, OH 45268.

Address correspondence to Joseph M. Quattro at the address above, or e-mail: quattro@biol.sc.edu.

Abstract

DNA sequence variation at a mitochondrial and a nuclear intron locus was surveyed within and among multiple populations of the inland silverside (*Menidia beryllina*) from the southeastern United States and revealed discordant phylogenetic patterns but similar patterns of population genetic variation across nuclear and mitochondrial loci. Mitochondrial variation was geographically structured, with strongly supported monophyletic assemblages among Gulf of Mexico population samples and a close association of the St John’s River (SJ) population with these same samples. Nuclear alleles were not strongly structured geographically, with little support for monophyly within or across basins. Conversely, population genetic parameters indicate that the bulk of genetic diversity for both genomes resides within and among Gulf of Mexico populations and that diversity within the Atlantic is largely restricted to the SJ population. The contrast in genetic variation and population phylogenies appears to be a function of historical demographic processes, most likely directed by fluctuating geomorphology of the Florida peninsula in response to North American glaciation cycles.

Key words: historical demography, mtDNA, nDNA, phylogeography, silverside

Quaternary ice ages greatly influenced population dynamics and demographics of large segments of the North American biota via cycles of advancing and retreating ice sheets (Hewitt 2000, 2004). Much of the terrestrial and freshwater biota of the southeastern United States was largely unaffected, as leading glacial edges did not extend into the region. Consequently, species inhabiting the southeastern United States persisted within relatively static geologic environments throughout the Quaternary (Jones et al. 2006). In contrast to inland environments, southeastern US coastal margins were indirectly and severely affected by Quaternary glaciation, which resulted in dramatic sea-level fluctuations that altered coastal margins (Scott 1997). The Florida peninsula was particularly affected, with glacial maxima (sea-level minima) producing a broad peninsula characterized by well-defined terrestrial habitats and glacial minima (sea-level maxima) submerging large sections of the peninsula thereby creating a series of islands (Arthur et al. 1994).

Genetic signatures of fluctuating coastal margins have been detected in species that have persisted through glacial oscillations, reflecting the demographic impact of the changes (Chenoweth et al. 1998; Knowlton and Weigt 1998; Williams et al. 2002; Reid et al. 2006). A concordant population-level genetic discontinuity along Florida’s eastern coast has been recovered largely in mitochondrial DNA (mtDNA) distributions and genealogies of codistributed native species (reviewed in Avise 2000; Soltis et al. 2006). Whereas phylogenetic and population genetic cytonuclear concordance has been commonly documented for inland southeastern US fish species (Quattro et al. 2001; Krabbenhoft et al. 2008), expectations for cytonuclear diversity for regionally sympatric coastal fishes are not as predictable due to demographic perturbations caused by Quaternary sea-level fluctuations.

Comparing intraspecific cytonuclear diversity can yield greater insight into the effects of complex historical demographics on population genetic diversity than surveys based solely on mtDNA. However, interpretation of genome-specific patterns of genetic diversity is complicated by the many differences in theoretically predicted behavior.
of alleles within each genome (Moore 1995) and by the putatively confounding effects of social and geographical structure and fluctuations in population sizes (Hoelzer et al. 1998). The southeastern United States’ historically dynamic coastal margins present an ideal opportunity to explore and compare genome-specific evolutionary signatures within species known to have persisted through such events.

This study uses variation in mtDNA and a nuclear intron to examine the impact of geological processes on historical demography as measured by genetic diversity and structure of southeastern US populations of the inland silverside (*Menidia beryllina*), a species that is widely distributed along the coastal margins of the Western Atlantic and Gulf of Mexico. Comparison of genetic variation among loci reveals a complex demographic history that appears to have been influenced by Quaternary sea-level fluctuations.

**Materials and Methods**

We collected inland silversides from 8 locations, 3 Gulf of Mexico and 5 Atlantic Ocean populations (Figure 1). Total genomic DNA was extracted from approximately 25–50 mg of excised caudal peduncle muscle tissue using a Qiagen DNeasy® Tissue Kit. After extraction, the presence of total genomic DNA was confirmed visually via ethidium bromide–stained 1.5% agarose gel electrophoresis. Total genomic DNA extracts were used as template in separate bromide–stained 1.5% agarose gel electrophoresis. Total genomic DNA was confirmed visually via ethidium bromide–stained 1.5% agarose gel electrophoresis.

**Figure 1.** Sampling locations for all populations of *Menidia beryllina* from the Gulf of Mexico and Atlantic coast of the southeastern United States. LA, Lake Pontchartrain, Louisiana (*N* = 9); AP, Apalachicola Bay, Florida (*N* = 10); HR, Hillsborough River, Florida (*N* = 10); SJ, St. John’s River, Florida (*N* = 9); NI, North Inlet, South Carolina (*N* = 9), SS, Stump Sound, North Carolina (*N* = 10); PS, Pamlico Sound, North Carolina (*N* = 10), NR, Neuse River, North Carolina (*N* = 9).

PCR products were precipitated in a 20% polyethylene glycol/2.5 M NaCl mixture and subsequently cleaned each with 2 rounds of 70% ethanol washes. We used forward and reverse PCR primers as sequencing primers in separate reactions using the ABI BigDye® Terminator v. 3.1 Cycle Sequencing Kit following the manufacturer’s specifications. Completed sequencing reactions were washed with 80% (4 °C) ethanol and precipitated via centrifugation. Sequencing reactions were read by an ABI 377 automated sequencer. After sequencing, we exported each sequence file into Sequencher™ software (Gene Codes Corporation, Ann Arbor, MI) and made contigs of forward and reverse sequences from each individual. We checked all base calls for each contig by eye and exported contigs from Sequencher™ as text files for further genetic analyses.

For the nuclear genome, alleles in heterozygotes were identified via direct inference, that is, by their presence in other individuals that exhibited the alleles in the homozygous state. When this method failed, we cloned the PCR products using an Invitrogen™ TOPO Cloning Kit following manufacturer’s protocols. All clones were sequenced as described above, and alleles were identified directly. Sequences of all alleles recovered from *Menidia* populations are in GenBank (accession numbers FJ966290-FJ966320 for D-loop haplotypes and FJ966321-FJ966346 for β actin alleles).

We analyzed genetic variation by constructing separate, locus-specific data files and estimated genetic diversity summary statistics and Tajima’s *D* (Tajima 1989) and *D* values of Fu and Li (Fu and Li 1993) using *DnaSP* v. 4.10 software (Rozas et al. 2003). We used *Arlequin* v. 3.1 (Excoffier et al. 2005) for analyses of molecular variance (AMOVAs; Excoffier et al. 1992). Phylogenies were constructed via the neighbor-joining method (Saitou and Nei 1987) under a Kimura 2-parameter evolutionary model using *PAUP* v. 4.0b10 software (Swofford 2003), and nodal support was assessed with 500 bootstrap (Felsenstein 1985) iterations. We settled on *Menidia peninsulae* as the out-group species, but during the course of analysis, *Menidia extensa* and *Menidia menidia* were used as additional out-groups. Regardless of species, out-group choice did not affect tree topology for either locus.

**Results**

Estimates of variation within the mitochondrial genome were based on 351 base pairs of the D-loop, and nuclear
genome variation was sampled in 433 base pairs of the β actin locus. For D-loop sequences, inland silverside populations were largely characterized by private haplotypes (unique to individual populations). Among Atlantic Ocean populations, shared haplotypes were only recorded between the North Inlet (NI) and Stump Sound (SS) and the Pamlico Sound (PS) and Neuse River (NR) populations. Only 2 Gulf of Mexico populations, Apalachicola Bay and Hillsborough River, shared a haplotype.

Private alleles also characterized the actin genotype distributions for Gulf of Mexico and St John’s River (SJ) populations of *M. beryllina*, but alleles in general tended toward broader geographic distributions than their D-loop counterparts. For example, 1 β actin allele was distributed among the SJ, NI, SS, PS, NR, and Lake Pontchartrain (LA) populations. Also, allelic variation among populations of *M. beryllina* was concentrated in Gulf of Mexico populations and the SJ sample, a trend evident but less conspicuous among D-loop haplotypes.

The neighbor-joining tree for D-loop haplotypes depicted genetically structured populations of *M. beryllina* (Figure 2). The LA and SJ populations were reciprocally monophyletic, but the Atlantic basin was not, based on a well-supported (85%) sister group relationship between the SJ population and all Gulf of Mexico populations. However, all other Atlantic haplotypes sampled from the NI, SS, NR, and PS populations formed a monophyletic assemblage. In contrast to the D-loop tree, the β actin gene tree (Figure 3) exhibited no intraspecific structure. Furthermore, only 1 node within the tree was well supported (monophyly of *M. beryllina* alleles). Resolution within the tree was influenced by β actin variation within and among populations, including fixation of all Atlantic populations, except SJ, for an allele that was shared with SJ and 1 Gulf of Mexico population, LA.

Estimates of genetic diversity varied greatly between the Gulf of Mexico and Atlantic Ocean populations. Given the phylogenetic results, specifically position of the SJ population, genetic diversities for the Gulf of Mexico and Atlantic Ocean were estimated inclusive and exclusive of the SJ population (Table 1). D-loop and β actin diversity estimates for Gulf of Mexico populations were not affected by the SJ sample and were higher than for Atlantic Ocean populations, especially nucleon diversity (θ̂) and average number of nucleotide differences (k̄). Essentially, more haplotypes and alleles were found within the Gulf of Mexico, and these tended to differ at more sites than those from the Atlantic Ocean populations. Placement of the SJ population had a profound effect on genetic diversity estimates for the Atlantic Ocean populations. Excluding the SJ population resulted in decreases in nucleon diversity (θ̂), overall nucleotide diversity (π̄), total number of haplotypes and alleles, and average number of nucleotide differences (k̄) for both D-loop and β actin loci.

To determine whether selection shaped allelic and haplotype distributions, we estimated both Tajima’s D (Tajima 1989) and D values of Fu and Li (Fu and Li 1993)

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**Figure 2.** Neighbor-joining tree of D-loop haplotypes. Five hundred iterations of bootstrapping were performed, with values >70 shown. Values at branch tips indicate total number of haplotypes at each position.

**Figure 3.** Neighbor-joining tree of β actin alleles. Five hundred iterations of bootstrapping were performed, with values >70 shown. Values at branch tips indicate total number of alleles at each position.
for the Gulf of Mexico and Atlantic Ocean populations (Table 1). Although values for each estimate varied depending on the specific group analyzed, none of the results was significantly different from null hypotheses of neutrality. Therefore, we found no evidence that either locus was under selection in any population.

Based on AMOVA, genetic variation in *M. beryllina* is partitioned somewhat differently within loci for the Gulf of Mexico and Atlantic Ocean populations (Table 2). The greatest percentage of D-loop variation was in the among-groups (\(\Phi_{ST}\)) category versus the within-population category for the and \(\beta\) actin locus. The percentage of total D-loop variation partitioned into the among-group category increased 20\% on moving the SJ population from the Atlantic Ocean to the Gulf of Mexico. The increase was attributable to a ~20\% loss from the among-populations within-groups category. Including the SJ population in the Gulf of Mexico basin also decreased the among-populations within-group category substantially. Genetic partitioning of the \(\beta\) actin locus was stable relative to alternative placements of the SJ population. Approximately 50\% of the nuclear \(\beta\) actin variation was in the within-population category, and ~25\% of the total variance was accounted for by each of the other 2 partitions in both hierarchies.

**Discussion**

Discordant evolutionary relationships and population genetic structure were recovered for *M. beryllina*. Strong genetic structuring of D-loop haplotypes is contrasted by a \(\beta\) actin phylogeny that revealed little, if any, structure among populations. Selection, a factor that Karl and Avise (1992) invoked to explain discordant results from nuclear and mtDNA markers in Atlantic Coast populations of American oyster (*Crassostrea virginica*), does not appear to have shaped D-loop or \(\beta\) actin variation, but the power to detect selection is limited by the number of loci surveyed and sample sizes for *M. beryllina*. Further, the biology of *M. beryllina* seems incompatible with male-dominated gene flow, which has been used to explain phylogenetic discordance in a diversity of organisms, including several marine species (Schrey and Heist 2003; Roberts et al. 2004). The inland silverside is a small, schooling fish known only to migrate in

### Table 1. Population genetic summary statistics for all *Menidia beryllina* populations grouped by region

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>(n_{tot})</th>
<th>(n_{haps})</th>
<th>(S)</th>
<th>(h)</th>
<th>(\pi)</th>
<th>(k)</th>
<th>(D_{Tajima})</th>
<th>(D_{Fu and Li})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulf of Mexico</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-loop (+) SJ pop.</td>
<td>4</td>
<td>38</td>
<td>23</td>
<td>41</td>
<td>0.952</td>
<td>0.033</td>
<td>10.7</td>
<td>0.010</td>
<td>0.170</td>
</tr>
<tr>
<td>D-loop (-) SJ pop.</td>
<td>3</td>
<td>29</td>
<td>17</td>
<td>38</td>
<td>0.941</td>
<td>0.035</td>
<td>11.9</td>
<td>0.522</td>
<td>0.630</td>
</tr>
<tr>
<td>(\beta) Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+) SJ pop.</td>
<td>4</td>
<td>76</td>
<td>24</td>
<td>8</td>
<td>0.817</td>
<td>0.006</td>
<td>2.45</td>
<td>1.26</td>
<td>1.28</td>
</tr>
<tr>
<td>(-) SJ pop.</td>
<td>3</td>
<td>58</td>
<td>14</td>
<td>6</td>
<td>0.727</td>
<td>0.005</td>
<td>1.43</td>
<td>1.89</td>
<td>1.16</td>
</tr>
<tr>
<td>Atlantic Ocean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-loop (+) SJ pop.</td>
<td>5</td>
<td>47</td>
<td>13</td>
<td>19</td>
<td>0.780</td>
<td>0.020</td>
<td>5.42</td>
<td>0.634</td>
<td>0.228</td>
</tr>
<tr>
<td>D-loop (-) SJ pop.</td>
<td>4</td>
<td>38</td>
<td>6</td>
<td>5</td>
<td>0.674</td>
<td>0.004</td>
<td>1.10</td>
<td>0.364</td>
<td>-0.024</td>
</tr>
<tr>
<td>(\beta) Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+) SJ pop.</td>
<td>5</td>
<td>94</td>
<td>13</td>
<td>6</td>
<td>0.329</td>
<td>0.002</td>
<td>0.897</td>
<td>-0.533</td>
<td>1.12</td>
</tr>
<tr>
<td>(-) SJ pop.</td>
<td>4</td>
<td>76</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(N\), number of populations; \(n_{tot}\), total number of haplotypes/alleles; \(n_{haps}\), number of unique haplotypes/alleles; \(S\), number of segregating sites; \(h\), nucleon diversity; \(\pi\), nucleotide diversity; \(k\), average number of nucleotide differences; —, not calculable due to absence of variation. All test of selection values failed to reject the null hypothesis of neutrality (\(P > 0.05\)).

### Table 2. Results of the AMOVA analysis

<table>
<thead>
<tr>
<th>Hierarchical level of comparison</th>
<th>Atlantic Ocean versus Gulf of Mexico</th>
<th>Atlantic Ocean (-SJ) versus Gulf of Mexico (+SJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-loop</td>
<td>(\beta) Actin</td>
</tr>
<tr>
<td>Within populations</td>
<td>3.60</td>
<td>50.3</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>36.4</td>
<td>25.0</td>
</tr>
<tr>
<td>Among groups ((\Phi_{ST}))</td>
<td>60.0</td>
<td>24.7</td>
</tr>
</tbody>
</table>

Estimates of genetic variance (\(\Phi\) statistics) are listed in percentages of total variation for each locus. Under the hierarchical level of comparison column, “populations” are defined by sampling location and “groups” are defined geographically by basin (i.e., Atlantic Ocean and Gulf of Mexico).
response to variation in zooplankton density (Bengston 1985; Wurtsbaugh and Li 1985).

Phylogenetic structure among D-loop haplotypes is consistent with neutral genetic variation molded by the Quaternary geomorphology of peninsular Florida. At sea-level minima and glacial maxima, peninsular Florida was a contiguous, highly exposed land mass. Conversely, the peninsula was a largely submerged oceanic platform with discontinuous exposed landmasses at glacial minima (Figure 4A). During these interglacial periods, only the central Floridian highlands pierced the ocean’s surface, producing a series of islands located between the southeastern US Atlantic coast and the Gulf of Mexico (Arthur et al. 1994). Gene flow across the largely submerged peninsula is consistent with the D-loop phylogeny in which the SJ population is placed with Gulf of Mexico populations. Similarly, the SJ population shares 2 \( \beta \) actin alleles with Gulf of Mexico populations and only 1 with Atlantic populations.

Although the D-loop and \( \beta \) actin loci have a similar number of alleles, their frequencies and distributions differ appreciably. Assuming gene flow from Gulf of Mexico to Atlantic populations due to rising sea levels and reduced peninsular landmass, gene tree discordance is most likely due to differential fixation times between mitochondrial and nuclear genomes. Using coalescence (Wakeley 2008), Rosenberg (2003) derived genealogical tree shape probabilities among recently separated taxa and found that polyphyly was initially most likely. As time since separation accumulated, the probability of paraphyly was maximal, followed by reciprocal monophyly. Mitochondrial haplotypes are expected to transition among these states faster than nuclear alleles due to the mtDNA genome’s 4-fold increase in coalescence time (Moore 1995; Rosenberg 2003). Such progress toward monophyly, particularly in the mitochondrial genome, is consistent with vicariant separation of Atlantic and Gulf of Mexico populations after reemergence of the Florida peninsula. *Menidia beryllina* is in an evolutionarily dynamic phase in which populations are nearly reciprocally monophyletic for mtDNA haplotypes but polyphyletic for nuclear alleles. Furthermore, under neutrality, a 4-fold difference in effective population size \( (N_e) \) between mitochondrial \( (N_f) \) and nuclear \( (2N_e) \) genomes is expected in estimators of population subdivision (Takahata 1993). When the SJ population is placed within the Gulf of Mexico, the \( \Phi_{ST} \) ratio approaches this theoretical disparity, thereby lending further support for governance of neutral evolution.

Genetic structure and patterns of diversity within and across genomes are consistent with a Gulf of Mexico ancestral distribution for *M. beryllina*. This argument was first proffered by Gosline (1948) based on morphometric and meristic variation within *M. beryllina* and was subsequently supported by analyses of allozyme variation. Johnson (1974,

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**Figure 4.** Geological fluctuations of the Florida peninsula and demographic history of *Menidia beryllina* inferred from the combined phylogenetic and population genetic results of the mtDNA and nDNA loci. (A) Geographic location of *M. beryllina* (gray oval) during glacial maxima of the Quaternary period. *Menidia beryllina* was confined to latitudes defining the Gulf of Mexico and the submerged Florida peninsula. Due to sea-level fluctuations caused by glacial advance and retreat, the Florida peninsula was, at times, characterized only by a series of islands consisting of the peninsula’s highest elevations. Figure courtesy of Ed Laine and Frank Rupert and used with permission. (B) After recession of the most recent Wisconsinan glaciation, peninsular Florida emerged and occupied its current geomorphology. The ancestral *M. beryllina* populations split (shaded ovals), resulting in allopatry. Cut off from populations within the Gulf of Mexico, individuals on the east coast of Florida then migrated northward along the Atlantic Coast of the southeastern United States (solid arrow).
1975) found that inland silverside allozyme diversity was characterized by a well-defined Gulf/Atlantic disjunction, much like our data, and structured latitudinally, which was interpreted as evidence of selection shaping patterns of diversity within *M. beryllina*. Interestingly, southeastern US populations of *M. beryllina* were more variable for allozyme loci than the D-loop and β actin intron loci. Although we found no evidence for selection, incongruence between studies may be a function of marker choice. Variation in some proteins (e.g., allozymes) may be controlled largely by selection in various species, not by the stochastic forces expected to govern neutral loci (e.g., introns). Interestingly, patterns of genetic diversity in 1 allozyme locus (*Phi*-*A*) reported by Johnson (1974) are comparable to β actin intron variation, perhaps reflecting the signature of historical demography.

The β actin and D-loop gene trees and variation also support Gosline’s (1948) hypothesis of recent isolation of eastern Florida populations from Gulf of Mexico con specifics, with subsequent colonization of the Atlantic coast. If the SJ population is placed with Gulf of Mexico populations, as indicated by the mtDNA tree, haplotype diversity among Atlantic populations decreases significantly and allelic variation is eliminated. All other measures of genetic diversity decrease similarly. Such decreases are hallmarks of a genetic bottleneck (Nei et al. 1975; Maruyama and Fuerst 1985). The magnitude of reductions in genetic diversity observed within each genome also indicates that bottleneck duration or the reduction in effective population size (*N_e*) as a result of the event (or a combination of these 2 factors) must have been rather severe. Therefore, analyses of genetic variation strongly suggest that southeastern US Atlantic populations north of the SJ were established via founders emigrating from Gulf of Mexico populations (inclusive of the SJ population; Figure 4B).

In summary, D-loop and β actin sequence variation and gene trees indicate that the evolutionary and demographic history of *M. beryllina* has been strongly influenced by fluctuating sea levels associated with Quaternary glaciation cycles. Genetic signatures of historical demographic events are evident within both D-loop and β actin, and cytonuclear comparisons have revealed a complex evolutionary history for this fish.

**Funding**

South Carolina Sea Grant (No. R/MT-5); Cooperative Institute for Fisheries Molecular Biology (FISHTIE) Grant (No. R/FT-1B) to J.M.Q.

**Acknowledgments**

We thank the Florida Fish and Wildlife Conservation Commission, the Belle W. Baruch Institute for Coastal and Marine Sciences, and Fred C. Rohde for collection of *M. beryllina*. We also thank John Darling, Shaoxing Ding, Adena Leibman, and Mark Roberts for constructive comments on earlier versions of the manuscript.

**References**


Received August 12, 2008; Revised March 31, 2009; Accepted April 9, 2009

Corresponding Editor: Robin Waples