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research paper

Population genetics and geometric morphometrics of the key silverside, *Menidia conchorum*, a marine fish in a highly-fragmented, inland habitat

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ABSTRACT.—Gene flow between populations is restricted in fragmented habitats, which can promote a more rapid genetic diversification as evolutionary forces act on isolated gene pools. Here, we compare two silverside species occurring in a continuous coastal habitat (tidewater silverside, *Menidia peninsulae* Goode and Bean, 1879) vs a highly fragmented habitat consisting of hypersaline pools (key silverside, *Menidia conchorum* Hildebrand and Ginsburg, 1927). First, we aim to better resolve the morphological and genetic relationships between the two nominal species using a haplotype analysis at two loci (NADH dehydrogenase 2, internal transcribed space r2) and geometric morphometrics. Despite some haplotype sharing and incomplete lineage sorting, they were both genetically differentiated (Φ_{ST} = 0.2186 and 0.4198, respectively, P < 0.0001) and showed significant discriminating morphometric characteristics. Second, we made a temporal comparison of genetic diversity and effective population size (N*e*) in key silversides over time using seven polymorphic microsatellite loci and found that genetic diversity and N*e* of key silversides were lower in these pools compared to tidewater silversides in the marine environment and decreased over time. We conclude that key silversides more likely represent an ecotype of the tidewater silverside and thus harbor a distinct adaptive potential. Further, our results support the hypothesis that highly fragmented aquatic habitats promote rapid genetic change and species diversification. Finally, we discuss the management applications of our study pertaining to the current listing of key silversides as a National Marine Fisheries Service Species of Concern.

Highly-fragmented habitats have often been shown to harbor higher biodiversity compared to continuous habitats (Stratham 1990, May 1994, Ward et al. 1994). Gene flow is more easily maintained between populations in continuous habitats and tends to homogenize genetic diversity. In contrast, populations living in fragmented habitats generally consist of smaller, more isolated gene pools exposed to higher levels of genetic drift resulting in more rapid genetic change. In addition, small, local populations can also undergo genetic differentiation through adaptation to local environmental conditions. The culmination of these processes promote differentiation and speciation in highly fragmented habitats (Stratham 1990, Barraclough et al. 1998, Puebla 2009). In aquatic systems, highly fragmented habitats are more frequently found in freshwater systems such as rivers and lakes compared to marine systems, which generally form continuous habitats with few barriers to gene flow. For example, Bloom et al. (2013) invoked habitat fragmentation to explain higher diversification rates among freshwater New World silversides (Teleostei, Atherinopsidae), which frequently inhabit highly-fragmented river systems compared to marine species living in continuous coastal environments. Similarly, Atlantic silversides (*Menidia menidia* Linnaeus, 1766) living in a continuous coastal habitat along the East Coast of the United States of America exhibit genetic homogeneity over a wide geographic area (Conover 1998), which is consistent with this hypothesis. However, no studies have actually compared the population genetics of silversides inhabiting continuous habitats to populations in a highly-fragmented habitat to determine if the populations are indeed smaller with lower levels of genetic diversity, and exhibit effects of genetic drift and non-random mating (inbreeding), such as deviations from Hardy-Weinberg due to an excess level of homozygosity.

The tidewater silverside, *Menidia peninsulae* Goode and Bean, 1879, is widely distributed in the Gulf of Mexico inhabiting a continuous coastal habitat. In contrast, the key silverside *Menidia conchorum* Hildebrand and Ginsburg, 1927, is endemic to inland hypersaline pools in the Florida Keys (Getter 1981, Duggins et al. 1986, Conover et al. 2000). The taxonomic status of the key silverside is not clear, indeed, due to a lack of clear diagnostic morphological features separating them it has been suggested that key silversides are an ecotype of the tidewater silverside (Duggins et al. 1986, Conover et al. 2000). An ecotype is a subdivision below the species level, which is adapted to specific environmental conditions found in a distinct habitat and distinguished by morphological, genetic, and physiological differences (Wilson and Brown 1953, Begon 2006). Resolving the genetic characteristics of key silverside populations is important as it is a Species of Concern for potential listing under the US Endangered Species Act, due mainly to threats to its limited habitat (status review available at: http://myfwc.com/media/2273331/Key-Silverside-BSR.pdf).

The highly-fragmented habitat of the key silversides offer a unique opportunity to determine the effect on genetic and species level when a continuously distributed species enters a more fragmented, inland habitat, and to compare genetic diversity and effective population size (N*e*) to populations of a closely related (possible) parent species inhabiting a continuous habitat. The individual pools are shallow (typically <1 m depth) and small [mean size = 0.27 (SD 0.33) km^2], have a high salinity (34–48), and are separated by a geographic barrier (land), precluding contemporary gene flow through unassisted migration. They are exposed to random fluctuations in environmental conditions resulting in water levels and salinity fluctuating markedly due to evaporation and precipitation (Getter 1981, Conover et al. 2000). In this setting, each

pool would be expected to harbor a relatively small, isolated population that is prone to genetic drift and inbreeding, two processes that would increase homozygosity and decrease overall genetic diversity. The extreme shift in environmental conditions (salinity) could result in a change in selective pressures that could also drive rapid evolution and further reduce genetic diversity.

Here, we compare two nominal silverside species that occur in two aquatic habitats that differ greatly in their degree of fragmentation with two objectives. First, we aim to better resolve the genetic and morphological relationship between the key and tidewater silversides. We do this by performing a haplotype analysis of mitochondrial and nuclear loci to determine the levels of genetic divergence between the two nominal species and identify patterns of lineage sorting. While the taxonomic status of the key and tidewater silverside is unclear, both species have been shown to be genetically and morphologically distinct species compared to a third silverside species found in Florida, the inland silverside, *Menidia beryllina* (Cope, 1867) (Bloom et al. 2009). We included sequences obtained from the inland silversides in the haplotype analysis to compare levels of divergence and patterns of lineage sorting, similar to including an outgroup in a phylogenetic study. In addition, we analyzed the body shape of these species to test for significant morphometric variation, which we hypothesized should exist between these nominal species. Second, we used seven polymorphic microsatellite loci to track the genetic diversity and N*e* of key silversides over time to determine if they have small, isolated, and rapidly-changing gene pools. We measured population divergence and assessed changes in genetic diversity in 1999 compared to 2012, and compared their genetic diversity to their hypothesized parent species, the tidewater silverside.

Methods

Field Sampling.—Archived key silversides used in the present study were collected by Conover et al. (2000) in February 1999 from 18 different hypersaline pools in the Florida Keys. Archived key silverside samples from two Florida Keys locations [Sugarloaf Key (SK), Grassy Key (GK); see Fig. 1 for all site locations] and inland silversides from one location [Crocodile Lakes (CL)] were used for haplotype analysis. Nine Florida Keys sample sites were revisited and sampled in February 2012 using a 30 × 2m beach seine, the three remaining locations sampled in 1999 were no longer accessible with a beach-seine at this time. In the sampled pools, the number of key silversides recovered ranged from 2 to more than 100; a mean of 44 individuals were caught per haul. Salinity ranged from 37.2 to 46, with the exception of Key Largo, which had the lowest salinity at 33.1. Key silversides were identified and frozen prior to genetic and morphometric analysis. Tidewater silverside samples were obtained from two large Gulf of Mexico estuaries: Charlotte Harbor (CH) and Sarasota Bay (SB). Detailed gear description and sampling protocols can be found in Poulakis et al. (2003).

Objective I: Genetic and Morphological Relationships.—DNA was extracted from 15 to 30 mg of tissue using a standard commercial kit (DNeasy, Qiagen, Valencia, California). We amplified one mitochondrial locus, NADH dehydrogenase 2 (ND2), and one nuclear locus, internal transcription spacer2 (ITS2). ND2 was used in a previous study focused on the delineation of species of *Menidia* (Bloom et al.

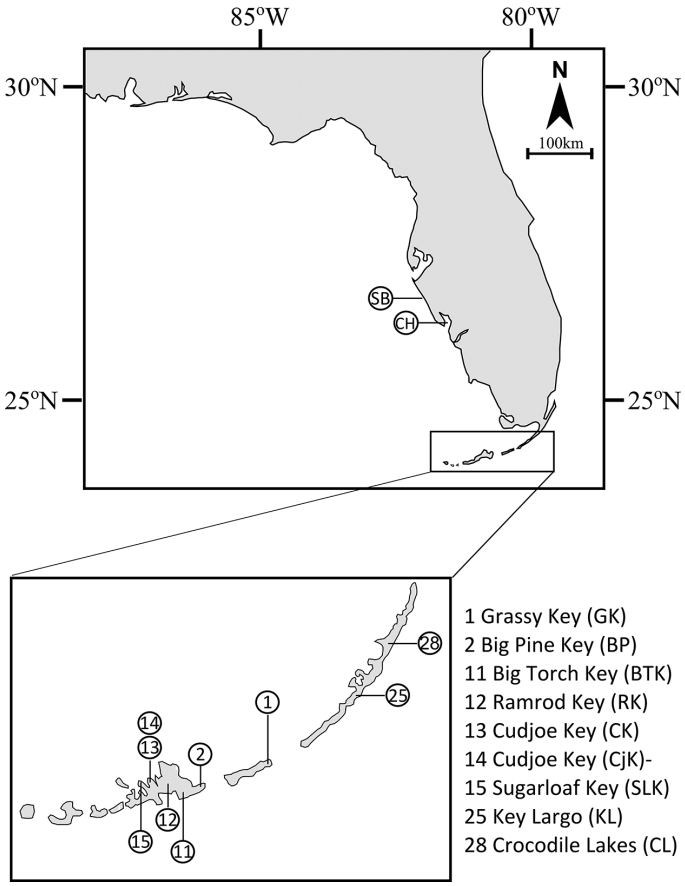

Figure 1. Sample locations for *Menidia conchorum* and *Menidia peninsulae* off the Florida Keys and the Gulf of Mexico. Location numbers correspond to Conover et al. (2000) report. Abbreviations in parenthesis are used throughout text, figures, and tables.

2009). Primers for ND2 (MenND2F: 5′-TACTATAATTACCCTCGCCCTAGCC-3′, MenND2R: 5′-GTAGAGAAGGTTATGATGAAGTAGG-3′) were designed using sequences of the mitochondrial genome of the Atlantic silverside, *Menidia menidia* (Accession number: AB370893.1). To design primers for the ITS2, we initially used universal primers (Plank et al. 2010) and then designed internal primers using successfully amplified sequences (MenITS2F: 5′-GCAGGACACATTGATCATCGA-3′, MenITS2R: 5′-TCGGCAAGAGAGGGAGAGAC-3′). All loci were amplified in 50-µl volumes containing genomic DNA, 1X PCR buffer (Qiagen Inc., Valencia, California), 40 µM dNTPs, 12.5 µmol of each of the primers and Taq polymerase. Thermal cycling was conducted for 35 cycles of 1 min at 95 °C, 1 min at the primer-specific annealing temperature T_a (ND2: 52 °C; ITS2: 55 °C) and 1 min at 72 °C, and concluded with a final extension step of 72 °C for 10 min. PCR products were purified and sequenced on ABI 3730 DNA analyzer.

All distinct sequences were verified by sequencing them in both the forward and reverse direction. Next, sequences were individually checked for quality, trimmed and imported into ClustalX (Thompson et al. 2002) for alignment. The alignment was exported into Arlequin v3.5 (Excoffier and Lischer 2010) to identify and

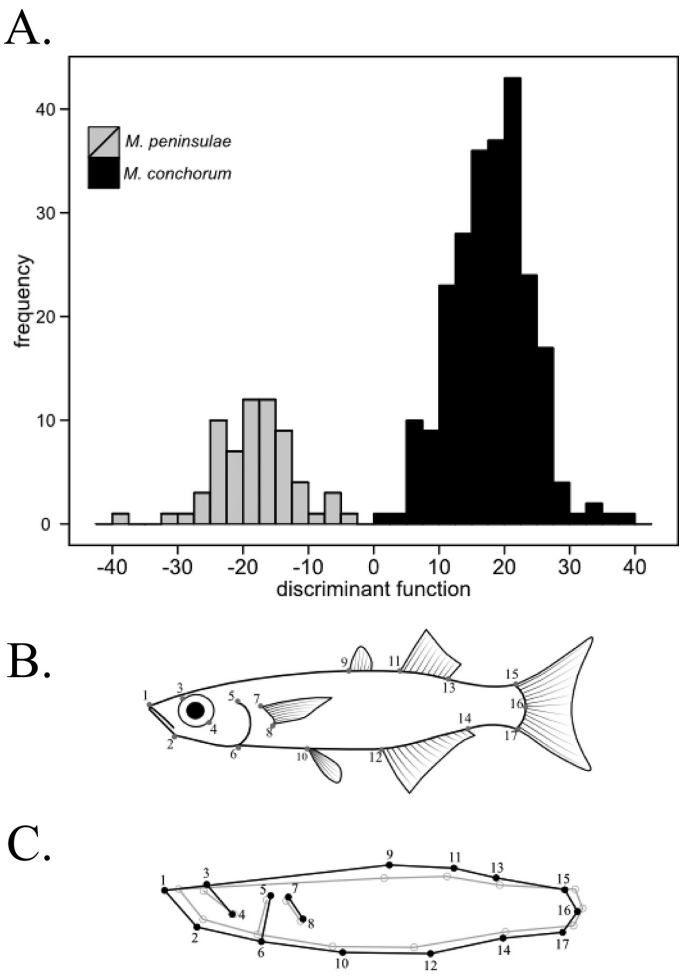

Figure 2. Shape differences between *Menidia peninsulae* and *Menidia conchorum*. (A) Frequency of observations along a discriminant function defining shape differences between *M. peninsulae* (gray) and *M. conchorum* (black). (B) Diagram of landmarks used in geometric morphometrics. (C) Mean shapes of groups using same coloring scheme as above (differences are scaled by a factor of two for visualization).

characterize haplotypes and their relationships using the Tamura and Nei model and calculate Φ_{ST} as a measure of genetic differentiation between key, tidewater, and inland silversides. A haplotype network was established using TCS v2.1 (Clement et al. 2000) based on statistical parsimony. For morphometric comparisons, we combined tidewater silversides from two Gulf of Mexico sites (n = 65), and key silversides from seven Florida Key locations (n = 238). To reduce a potential source of bias relating to size, we only included individuals over similar centroid size ranges in both groups. We placed specimens with their right lateral side facing upward and photographed them with a digital camera set on a tripod. Next, we used tpsDIG2 (Rohlf 2010) to digitize 17 landmarks on each individual following Fluker et al. (2011) (Fig. 2). Once digitized, shapes were aligned using Generalized Procrustes Analysis (GPA) in MorphoJ (Klingenberg 2011). GPA is a least-squares superimposition procedure

that aligns specimens by scaling, rotating, and translating their shapes, as defined by a set of homologous landmarks (Zelditch et al. 2004).

Discriminate function analysis (DFA) embedded in MorphoJ (Klingenberg 2011) was conducted to identify shape differences between key and tidewater silversides. We performed a multivariate regression of shape on centroid size to test for residual size effects and compared groups with a MANCOVA in tpsRegr (Rohlf 2011).

Objective II: Genetic Diversity and N*e* Over Time.—Seven microsatellite loci were amplified in 469 key silversides from seven pools for which we had a sufficient sample size from both 1999 and 2012 to estimate genetic diversity (Hale et al. 2012). One-hundred-and-eight tidewater silversides were genotyped from Charlotte Harbor and Sarasota Bay for comparative purposes. Microsatellite loci (Sbrocco and Barber 2011) were amplified in a 10-µl reaction containing genomic DNA, 1×PCR buffer, 10× bovine serum albumin, 1.5–3.5mM $MgCl_2$, 0.12 mM dNTPs, 0.16 µM of the reverse primer and fluorescently labeled m13 primer (Schuelke 2000), 0.04 µM of the species specific forward primer, and 1 U Taq polymerase. Thermal cycling profiles consisted of 4 min at 94 °C followed by 30 cycles of 94 °C for 15 s, primer specific annealing temperature T_a for 15 s and 72 °C for 45 s, concluding with 5 cycles of 94 °C for 15 s, 53 °C for 15 s, and 45 s at 72 °C with a final extension at 72 °C for 10 min. The amplified products were run on an Applied Biosystems ABI3730 sequencer with an internal size standard (LIZ-500 Applied Biosystems). To reduce genotyping error, a series of quality control measures was implemented: (1) for each locus, a subset of eight were re-amplified at 3 °C below the primer-specific T_a to determine if reduced stringency would result in the amplification of null alleles, i.e. additional alleles amplified for individuals previously scored as homozygotes; (2) alleles were scored by a single analyst using Peakscanv1.0 (Applied Biosystems), and approximately 30% of genotypes were verified by a second analyst; and (3) we tested for null alleles, stutters, and large allelic dropouts using MicroChecker (Van Oosterhout et al. 2004).

The genetic diversity for each sample site for all key and tidewater silversides was characterized by calculating the expected and observed heterozygosity, H_e and H_o (Nei 1978), respectively, and the allelic richness as implemented in FSTAT (Goudet 1995). Exact tests implemented in Genepop (Raymond and Rousset 1995, Rousset 2008) were used to test for linkage disequilibrium and deviations from Hardy-Weinberg Equilibrium (HWE). To determine the effect of loci out of HWE on ability to detect population differentiation and determine the effective population size (N*e*), these general statistics were calculated including and excluding loci out of HWE. As these results were not substantially different in terms of overall pattern and significance, we included all loci in further analyses.

Spatial and temporal differentiation between and within key silverside sites were determined by estimating global and pairwise F_{ST} using FSTAT (Goudet 1995). In addition, we used Structure (Pritchard et al. 2000), a Bayesian clustering approach, to determine spatial and temporal clusters of individuals. Spatial structure was analyzed separately for pools in 1999 and in 2012. We simulated the number of clusters K = 1 to 10 for five independent runs, each to determine convergence for a burn-in period of 150,000 Markov chain Monte Carlo (MCMC) steps followed by 500,000 MCMC steps. Pritchard et al. (2000) suggest determining the convergence of the mean estimate of the ln probability of ΔK, which occurs at approximately seven for both 1999 and 2012 (Online Appendix 1). Further, we used the ΔK metric suggested

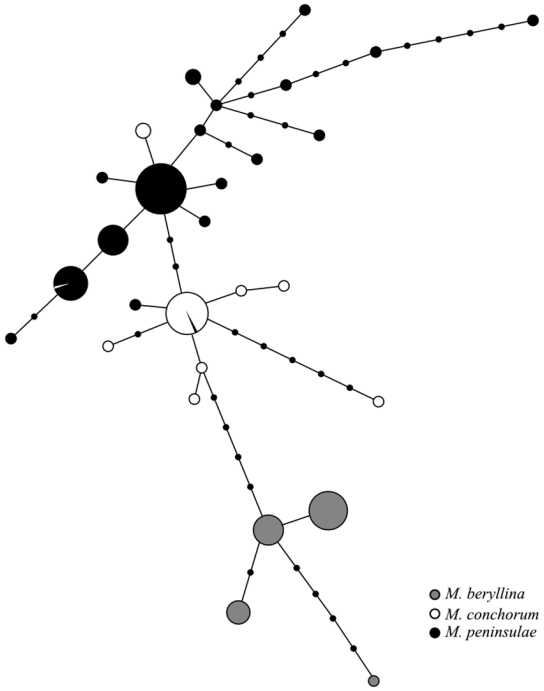

Figure 3. ITS2 haplotype network for nominal *Menidia* species examined off the Florida Keys. Size of circle is proportional to the frequency of each haplotype. Small solid circles represent extinct or unsampled haplotypes.

by Evanno et al. (2005) to determine the statistically most-supported number of clusters.

The inbreeding coefficient F_{IS} was calculated using FSTAT (Goudet 1995) to measure heterozygote deficiency resulting from inbreeding at the sample level. In addition, we used Storm (Frasier 2008) to calculate the internal relatedness (IR), which measures the level of relatedness of the parents of an individual (Amos et al. 2001). Outbred individuals will have an IR ≤ 0, while individuals derived from mating of related individuals would have a positive value, between 0 and 1.

Effective population size (N*e*) was estimated for each sample location and sampling time (i.e., 1999 and 2012) using the linkage disequilibrium method implemented in LDNe (Waples 2006, Waples and Do 2008) with a lowest included allele frequency p_{crit} = 0.03. This method estimates N*e* based on the small level of linkage of alleles occurring due to sampling error during recombination and only requires a single temporal sample.

Results

Objective 1: Genetic and Morphological Relationships.—As expected, in both the haplotype network for ND2 and ITS2, the inland silverside haplotypes form a distinct group that does not share any haplotypes with the other two silverside species. Further, the tidewater and key silversides are shown to be genetically differentiated in both haplotype networks despite some shared haplotypes. Ninety-six individuals were sequenced for a 267bp fragment of the ITS2 locus containing 31 polymorphic sites (Fig. 3). We identified two shared haplotypes between key (*n* =

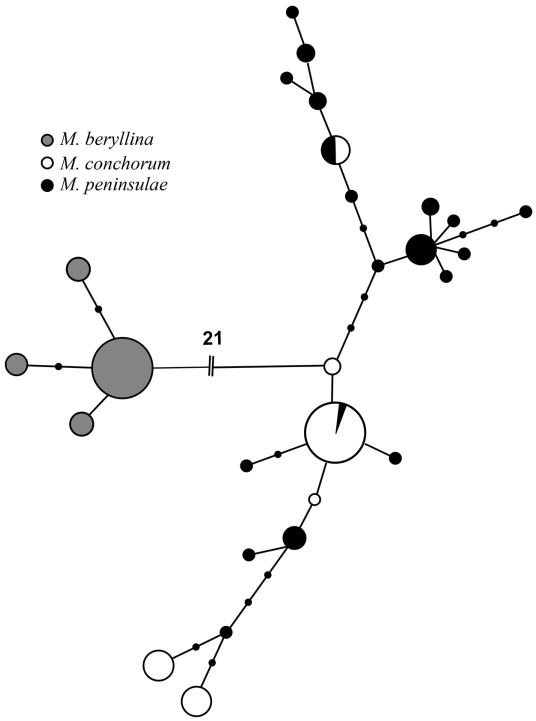

Figure 4. ND2 haplotype network for nominal *Menidia* species examined off the Florida Keys. Size of circle is proportional to the frequency of each haplotype. Small solid circles represent extinct or unsampled haplotypes.

45) and tidewater (n = 45) silversides at this locus, with an additional seven distinct haplotypes for keys silverside compared to 16 distinct haplotypes for tidewater silversides, respectively. Tidewater silversides had a haplotype within the key silverside branches of the network and vice versa. Four haplotypes were found for inland silversides (Fig. 3). There was significant differentiation between key and tidewater silversides (Φ_{ST} = 0.2186, P < 0.0001), but much stronger differentiation was determined between these two groups and the inland silverside (Φ_{ST} = 0.9132 for inland/tidewater silversides and Φ_{ST} = 0.8980 for inland/key silversides). We sequenced 124 individuals for 252bp of the mtND2 gene (27 polymorphic sites). Four inland silverside haplotypes, 19 tidewater silverside haplotypes, and 6 key silverside haplotypes were identified. There were two shared haplotypes between key and tidewater silversides (Fig. 4) and five tidewater silverside haplotypes were located within the key silverside branches of the network. Nonetheless, key and tidewater silversides were significantly differentiated (Φ_{ST} =0.4198, P < 0.0001), as were key/inland silversides (Φ_{ST} = 0.7388, P = 0.000) and tidewater/inland silversides (Φ_{ST} = 0.8339, P < 0.0001).

Discriminant function analysis based on 10,000 permutations indicated that the shape of key silversides was significantly distinguishable from that of tidewater silversides (P < 0.0001, Fig. 2A). Shape changes among groups were characterized by dorso-ventral deepening of the midsection and a more robust head in key silversides relative to tidewater silversides, resulting in an overall stouter appearance in the former (Fig. 2C). Additionally, there was a significant relationship between centroid size and shape (multivariate regression: P < 0.0001 on 10,000 permutations), but the rate

of shape change was marginally, although significantly, different among silverside groups (MANCOVA: $P = 0.044$). Because the slopes of individual groups were not homogenous, we could not use a single function to remove the effects of size and therefore could not follow up the test of slopes with a direct test of significance between groups.

As a consequence of our MANCOVA results, we performed a set of post hoc tests, where we created an independent categorical size factor by dividing the covariate (centroid size) into three groups of equal range (i.e., small, medium, and large). We performed additional DFAs comparing key and tidewater silversides in each group to verify that the overall direction of shape change was consistent across the size range of fish analyzed. The resulting DFAs based on 10,000 permutations, showed that there was significant discrimination among silverside groups in each size class ($n_{small} = 123$, $P_{small} < 0.0001$; $n_{medium} = 113$, $P_{medium} < 0.0001$; and $n_{large} = 67$, $P_{large} = 0.0001$). Further, visual comparisons of mean shapes for each group confirmed that the direction of change within size classes was consistent w ith overall shape variation between key and tidewater silversides (Online Appendix 2). Hence, the observed shape differences likely reflected true variation between silverside groups, not artifacts of size differences among samples.

Objective 2: Genetic diversity and N*e* Over Time.—Heterozygosity, allelic richness and F_{IS} for each locus at each key silverside pool sampled in 1999 and 2012 can be found in the supplementary material (Online Appendix 3, 4). In general, the heterozygosity ($H_o = 0.26–0.667$) and allelic richness ($A = 5.29–10.29$) of each key silverside population declined between the 1999 and 2012 (with the exception of CjK). There was no evidence of linkage disequilibrium between any pair of loci. Deviations from Hardy-Weinberg Equilibrium (HWE) due to an excess of homozygotes were detected at approximately 50% of all loci, sample-location, and sample-time combinations (112 combinations). For multi-locus comparisons, all tested populations were out of HWE with the exception of the Charlotte Harbor tidewater silversides and one key silverside population in one year (BTK in 1999). Microchecker did not detect evidence of stuttering or large allelic dropouts, though it did indicate that null alleles could be an issue based on the large discrepancies between expected and observed heterozygosities. We re-amplified a set of homozygotes for each locus to verify the genotypes and identify potential null alleles. This did not result in the amplification of a second allele in any instance.

Among the seven pools containing key silversides, pairwise F_{ST} for all comparisons between pools sampled in the same year (i.e., 1999 or 2012) and the same location between years were significant ($P < 0.001$) and remained significant after Bonferroni adjustment for multiple comparisons (Table 1). Bayesian inference of genetic structure is depicted in Figure 5, showing the individual membership coefficients for each cluster for selected values of K. Using the Evanno method, for the 1999 samples K = 3 was indicated to be the most likely number of clusters, with a second peak detected for K = 6. For the pools sampled in 2012, the strongest peak was detected for K = 5, with secondary peaks at K = 3 and K = 7 (Online Appendix 1).

When calculated over all loci, F_{IS} was positive and significantly greater than zero for all sample locations and sample periods, ranging from 0.233 to 0.514 with the exception of BTK in 1999 (0.041) and CK sampled in 2012 (0.166). The general pattern was an increase of F_{IS}, indicating an increase in non-random mating (inbreeding)

Table 1. Temporal and spatial analysis of *Menidia conchorum* population structure using pairwise F_{ST}. Pairwise F_{ST} of all seven pools sampled in 1999 are above the gray diagonal. Pairwise F_{ST} of all pools re-sampled in 2012 are below the gray diagonal. Temporal comparison for each pool is found along the gray diagonal. All values were significant to a level of $P < 0.001$ and remained significant after Bonferroni correction for multiple comparisons ($P < 0.05$). See Figure 1 for sample location abbreviation descriptions.

Location	BP	BTK	CjK	CK	KL	RK	SLK
BP	0.2804	0.1528	0.1658	0.1348	0.0872	0.3269	0.1312
BTK	0.1372	0.3561	0.1512	0.1595	0.1320	0.2635	0.1103
CjK	0.1213	0.3178	0.1561	0.1431	0.1556	0.2985	0.1050
CK	0.2093	0.3262	0.1365	0.1135	0.1242	0.2532	0.0886
KL	0.2537	0.3812	0.1785	0.1680	0.0532	0.2345	0.1290
RK	0.2099	0.3947	0.1535	0.1948	0.1966	0.2748	0.2632
SLK	0.3272	0.4488	0.2336	0.1836	0.198	0.182	0.1211

between 1999 and 2012, with the exception of CK and KL. Evidence of inbreeding was further corroborated through high-average IR values for sample pools (Fig. 6), and IR increased between 1999 and 2012 with the exception of CK and CjK. During both sample periods, levels of internal relatedness were high, ranging from 0.1291 to 0.5441 (SD 0.24–0.26) in 1999 and 0.3104–0.6623 (SD 0.26–0.30) in 2012. Overall the mean IR was 0.464, which is about what would be expected if parents of the average individual were related at the level of full siblings. Tests for pairwise relatedness did not indicate siblings or half-siblings present in the data set.

Estimates of N*e* (Table 2) for each key silverside population declined from 1999 (47.7–180.8) to 2012 (5.1–16.6), with the exception of one pool (CK) where N*e* increased from 253.9 to 645.2. In 1999, the confidence intervals (CI) also were much wider with the upper confidence limit being infinity in each case. In 2012, with the exception of CK, all pools had definitive CI limits. In contrast, the more widespread coastal tidewater silverside had a N*e* of 306.6 (with an upper CI limit of infinity). Not being able to get a bound CI for N*e* estimates is indicative of the sample size not being large enough compared to census size.

Discussion

More fragmented habitats have been shown to result in more isolated populations vulnerable to genetic drift and other stochastic processes and to thus promote more rapid genetic divergence. For example, Bloom et al. (2013) found that populations of silversides inhabiting the more fragmented habitats frequently found in freshwater systems had higher diversification rates compared to silverside species inhabiting continuous habitats encountered in the marine environment. Here, we compared two nominal silverside species inhabiting a highly-fragmented and continuous habitat by (1) determining genetic and morphological relationships between them and (2) analyzing changes in genetic diversity and N*e* over time.

Objective 1: Genetic and Morphological Relationships.—Key silversides are thought to be the result of an inland incursion by a parent species into highly-fragmented hypersaline pools in the Florida Keys (Getter 1981, Duggins et al. 1986, Conover et al. 2000). We found marked differences in the haplotype frequencies despite some haplotype sharing between key silverside and tidewater silverside

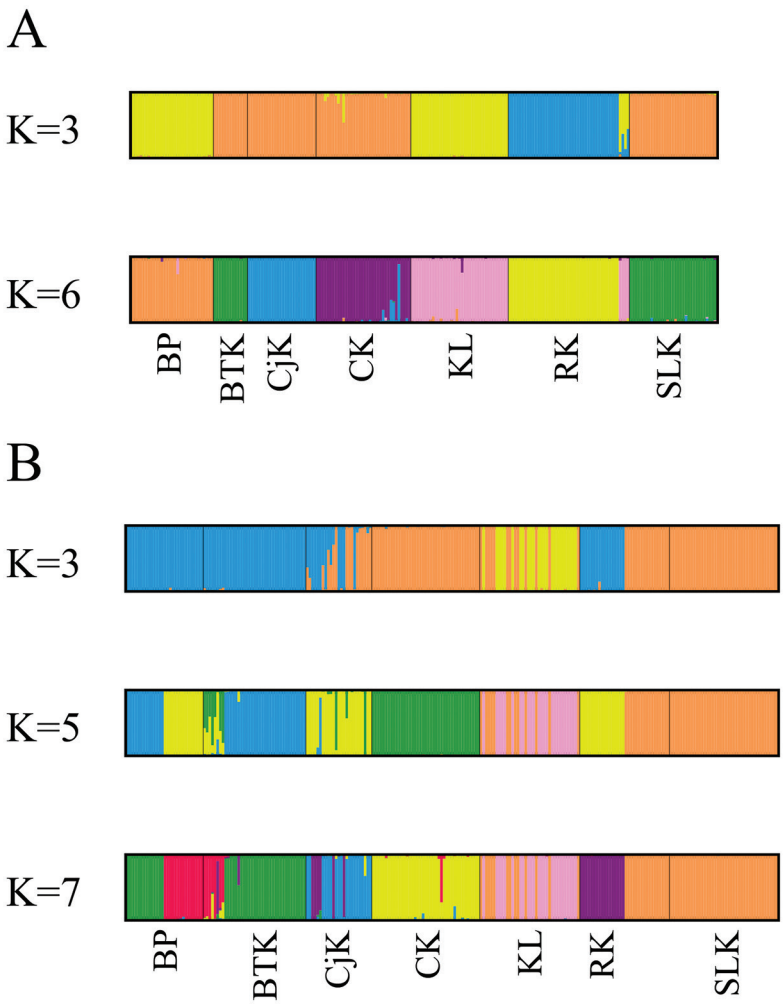

Figure 5. Bayesian inference of genetic structure for *Menidia conchorum* off the Florida Keys (see Figure 1 for sample location information). Structure results for no admixture model for K = 2–17 and sample locations (on *x*-axis) as a priori information. Individual membership coefficients for each cluster for (A) 1999 and (B) 2012 are represented by a single vertical line.

populations. Shared haplotypes could be indicative of recent gene flow, but given the enclosed nature of these pools we suggest that incomplete lineage sorting is more probable given the relatively young age of the Florida Keys in geologic time (emerged within the last 10,000 yrs; Hoffmeister and Multer 1968). Both the level of genetic divergence measured between these two groups and the presence of distinct haplotypes indicate that both key and tidewater silversides harbor a unique pool of genetic diversity and adaptive potential and have become evolutionarily independent since being geographically separated from each other. This genetic divergence is mirrored by significant morphometric differentiation. It is possible that morphological differences among groups in our study are partially due to differences in growth rates, as between the Atlantic and tidewater silversides, which display countergradient growth variation (Conover and Present 1990, Yamahira and Conover 2002,

Table 2. Estimates of *Menidia conchorum* effective population size (N*e*). N*e* estimates using the linkage disequilibrium method are shown for each sample location in 1999, 2012 and over both sample periods. Confidence interval (95%) estimated using jackknifing method. See Figure 1 for sample location abbreviation descriptions.

Sample location	1999	2012	Overall
BP	180.8 (33.7, ∞)	7.6 (4.4, 12.0)	11.1 (8.9, 13.6)
BTK	90.6 (77.5, ∞)	10.4 (2.8, 74.1)	29.5 (26.5, 32.7)
CjK	87.4 (54.6, ∞)	16.9 (6.7, 112.0)	31.9 (21.1, 53.7)
CK	253.9 (51.7, ∞)	645.2 (84.8, ∞)	145.1 (70.3, 1,381.0)
KL	72.5 (64.8, ∞)	16.5 (12.3, 22.5)	47.9 (35.8, 67.8)
RK	135.1 (1.4, ∞)	5.1 (2.9, 8.1)	25.4 (18.8, 35.6)
SLK	47.7 (17.2, ∞)	12.8 (2.8, 125.7)	17.5 (4.5, 78.7)
Menidia peninsulae		4,620.0 (143.9, ∞)	

Yamahira et al. 2007). Further, these morphological differences may be the combined result of divergence due to isolation and local adaptation to dissimilar habitats; fishes invading a new habitat are faced with a shift in environmental conditions and selective pressures that can trigger adaptive divergence and reduced gene flow, ultimately leading to speciation (Cracraft 1989, Beheregaray and Sunnucks 2001, Barluenga et al. 2006, Fluker et al. 2011, Bloom et al. 2013). Our results are consistent with the hypothesis that key silverside are an inland incursion by the closely-related tidewater silverside that has resulted in the formation of a genetically and morphologically distinct ecotype that is in the process of divergence into a distinct species.

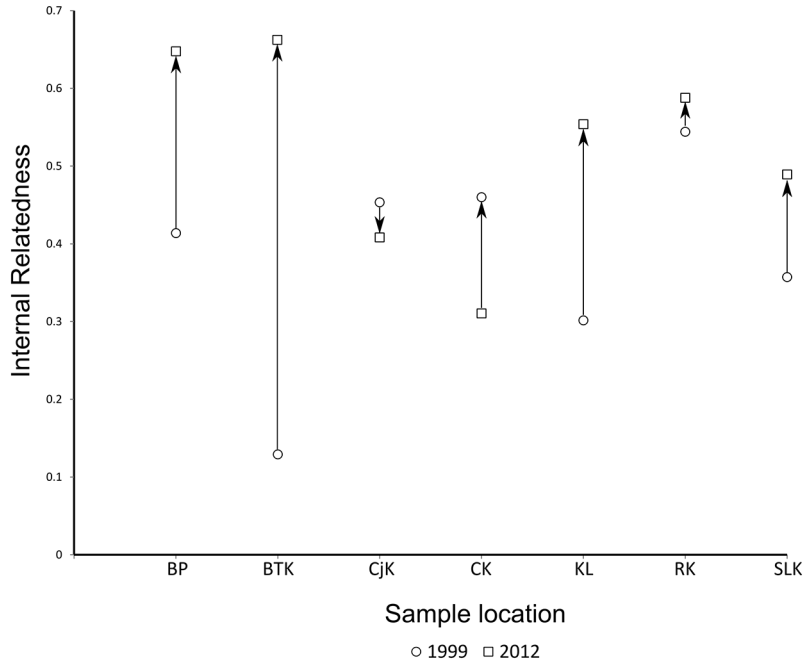

Figure 6. Internal Relatedness (IR) for all *Menidia conchorum* sample locations in 1999 and 2012. Trajectories of average IR-values for each sample location indicated by arrows. See Figure 1 for sample location information.

Objective 2: Genetic Diversity and N*e* Over Time.—Similar to the haplotype and morphometric analysis showing key and tidewater silversides to form two divergent groups each with their own distinct genetic and morphological characteristics, the microsatellite analysis of the key silversides revealed that this species is not panmictic. Rather, as is expected given the highly-fragmented nature of their habitat, each hypersaline pool forms an isolated population affected by stochastic and systemic processes with a shared overall trend of declining N*e* and genetic diversity. Although some of the pools were geographically relatively close to one another, it does not appear that adult fish or their eggs are transported between pools (e.g., during high tides, floods, or hurricanes) frequently enough to have any homogenizing effect. In contrast, we found modest differentiation between tidewater silversides sampled approximately 100 km apart in Sarasota Bay and Charlotte Harbor, two estuaries connected to the open ocean. Pairwise F_{ST} values between these two marine sites were an order of magnitude smaller than values between inland pool populations of key silversides separated by as little as 2 km. Similarly, genetic homogeneity over large spatial scales has also been observed among Atlantic silversides (*Menidia menidia*) along the United States of America eastern seaboard (Clarke et al. 2010).

One important limitation of our data set for assessing population structure, F_{IS}, and IR is that many loci were out of HWE for certain locus-population combinations due to an excess of homozygotes. Small, isolated populations should frequently exhibit this characteristic because they are prone to both genetic drift and inbreeding. We attribute the excess homozygosity observed in key silversides primarily to genetic drift and non-random mating, although null alleles could also be an issue in our data set. We suggest that null alleles are less important than drift and non-random mating based on several factors. First, reduced stringency (lower amplification temperatures) did not result in additional alleles being amplified. Second, if null alleles were an issue we would expect the problem to be more pronounced in the older samples due to degraded DNA, but deviations in HWE were more pronounced in more recent samples. Further, for each microsatellite locus there was at least one population that was not out of HWE and for these population/marker combinations Microchecker did not detect evidence of null alleles. If null alleles were indeed an issue, we would expect them to occur consistently across all populations. The calculation of pairwise F_{ST} is based on determining differences in allele frequencies among populations. Cluster analysis in Structure not only assumes HWE and linkage disequilibrium, it uses the metrics and how they affect allele frequencies to cluster individuals based on their genotypes. For both pairwise F_{ST} and cluster analysis in Structure will be affected by using loci out of HWE. We find that when loci out of HWE are eliminated from the analysis, F_{ST}, F_{IS}, and IR are lower, but still significant and overall exhibit the same pattern. Nevertheless, the violation of the assumption of HWE for many loci/population combinations suggests some of our results on population structure should be viewed with caution.

The existence of key silversides in the hypersaline pools of the Florida Keys has been documented since at least the 1920s (Hildebrand and Ginsburg 1927, Getter 1981), representing nearly 100 generations for this short-lived (annual), rapidly maturing species, assuming that they have continuously inhabited these pools. In the absence of gene flow between populations in different pools, genetic differences are expected to arise based on stochastic changes in allele frequencies due to genetic drift, which is inversely proportional to N*e*. Point estimates of N*e* were low in all

pools in both 1999 and 2012. Further, there was a distinct decline in N*e* in six of seven pools between 1999 and 2012. Notably, the upper confidence limit was infinity in all pools in 1999, but <100 in six of them by 2012, further indicating that the N*e* of each pool decreased in the 13 yrs between sampling events. The effective population size of tidewater silversides were larger in 2012 than all of the pools, frequently by 1–2 orders of magnitude, and the latter had an unbound upper confidence interval. The effective population sizes of key silversides are comparable to those found in highly structured freshwater fish populations, including the three-spined stickleback, *Gasterosteus aculeatus* Linneaus, 1758 (N*e* = 15–39, Araguas et al. 2012; N*e* = 12–86, Seymour et al. 2013), brown trout, *Salmo trutta* Linnaeus, 1758 (N*e* = 16–32, Sanz et al. 2011; N*e* = 63, Charlier et al. 2011), the Tokyo bitterling, *Tanakia tango* Tanaka, 1909 (N*e* = 5–28, Kubota et al. 2010), and the purple spotted Gudgeon, *Morgunda adspersa* Castelnau, 1878 (N*e* = 30–60, Hughes et al. 2012).

The estimated effective population sizes of key silversides in each of the pools we examined in 1999 and 2012 were at levels where they would be expected to be highly susceptible to genetic drift, which erodes genetic diversity (Frankham 1996, Lynch and Lande 1998). In all seven pools, we observed substantial changes in genetic diversity between 1999 and 2012. These changes were large enough that F_{ST} and Bayesian clustering analysis indicated that each pool population was significantly differentiated between the two sampling periods. In six of them, allelic richness and/or observed heterozygosity decreased while measures of inbreeding at the population and individual level increased. If null alleles were wholly responsible for excess homozygosity in these pools then null allele frequencies would have to have increased in these six pools simultaneously over time, which is unlikely. We cannot determine whether the reduction in genetic diversity observed in the other six pools occurred gradually over the entire 13 yrs or during specific discrete bottleneck events, such as a particularly dry year causing the pool size and silverside population to experience an unusually large decline. Nevertheless, either scenario is consistent with the Bloom et al. (2013) hypothesis that isolated populations of silversides occupying fragmented inland habitats are prone to rapid genetic change that could contribute to reproductive isolation and eventual allopatric speciation. In short-lived silversides, our study suggests measureable change in genetic diversity can occur over as little as 13 yrs. A similar result was found in Chinook salmon, *Oncorhynchus tschawytscha* Walbaum, 1792. After their introduction to the Great Lakes in the 1960s, significant population structure (F_{ST} = 0.036–0.133) was detected over fewer than 10 generations (Suk et al. 2012).

Management Applications.—The key silverside is currently listed as a Species of Concern based on its limited and diminishing habitat (http://myfwc.com/media/2273331/Key-Silverside-BSR.pdf) and is currently under review for a potential listing under the US Endangered Species Act. As part of this process it is important to determine the taxonomic status of the key silverside. Though our analysis of morphological and genetic relationships between key and tidewater silversides does not conclusively solve the species issue, it does support the hypothesis set forth by Conover et al. (2000) that key silversides are the result of an inland incursion and are best described as an ecotype of the tidewater silverside. While the key silverside may not constitute an independent species, it does represent a highly divergent group of populations harboring a unique genetic diversity and adaptive potential. Further, the

analysis of genetic diversity and N*e* show that each pool constitutes an independent population with a unique genetic diversity acted upon by evolutionary forces such as genetic drift. While a more in-depth phylogenetic analysis beyond the scope of this study is necessary to fully resolve the taxonomic status, our results point toward the necessity of managing key silversides as a separate management unit.

Summary and Outlook.—The inland incursion(s) of tidewater silversides into the Florida Keys has resulted in the key silverside ecotype, represented by a complex of disconnected populations with small N*e* and high levels of inbreeding, resulting in deviations from HWE. In contrast, tidewater silversides inhabiting large estuaries connected to the ocean have larger N*e* and much lower inbreeding levels. We show large reductions in all metrics of genetic diversity occurring in 6 of 7 key silverside populations over a 13-yr period, as well as an increase in inbreeding and reduction in N*e* over the same time period. Our results argue against key silversides forming one cohesive species in the future. Instead, the configuration of isolated, rapidly changing gene pools is more likely to form a species flock (Echelle and Kornfield 1984). Although genetic drift, selection, or bottlenecks occurring between the two sampling periods could individually or in combination explain the declines in genetic diversity, they are all consistent with the hypothesis that fish living in fragmented inland habitats have small, rapidly changing gene pools, which underpins their more rapid diversification when compared to fishes inhabiting more continuous habitats (Stratham 1990, May 1994, Benton 2001, Betancur 2010, Eschmeyer et al. 2010, Bloom et al. 2013).

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Literature Cited

Amos W, Worthington WJ, Fullard K, Burg TM, Croxall JP, Block D, Coulson T. 2001. The influence of parental relatedness on reproductive success. Proc Biol Sci. 268(1480):2021–2027. http://dx.doi.org/10.1098/rspb.2001.1751

Araguas RM, Vidal O, Pla C, Sanz N. 2012. High genetic diversity of the endangered Iberian three-spined stickleback (*Gasterosteus aculeatus*) at the Mediterranean edge of its range. Freshw Biol. 57(1):143–154. http://dx.doi.org/10.1111/j.1365-2427.2011.02705.x

Barluenga MKN, Stolting W, Salzburger W, Muschick M, Meyer A. 2006. Sympatric speciation in Nicaraguan crater lake cichlid fish. Nature. 439(7077):719–723. http://dx.doi.org/10.1038/nature04325

Barraclough TG, Vogler AP, Harvey PH. 1998. Revealing the factors that promote speciation. Phil Trans: Biol Sci. 353(1366):241–249. http://dx.doi.org/10.1098/rstb.1998.0206

Begon M. 2006. Ecology: from individuals to ecosystems. Blackwell Publishing 4th ed. p. 5–8.

Beheregaray LB, Sunnucks P. 2001. Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis.* Mol Ecol. 10(12):2849–2866. http://dx.doi.org/10.1046/j.1365-294X.2001.t01-1-01406.x

Benton MJ. 2001. Biodiversity on land and in the sea. Geol J. 36(3–4):211–230. http://dx.doi.org/10.1002/gj.877

Betancur R. 2010. Molecular phylogenetics supports multiple evolutionary transitions from marine to freshwater habitats in ariid catfishes. Mol Phylogenet Evol. 55(1):249–258. http://dx.doi.org/10.1016/j.ympev.2009.12.018

Bloom DD, Piller KR, Lyons J, Mercado-Silva N, Medina-Nava M. 2009. Systematics and biogeography of the silverside tribe Menidiini (Teleostomi: Atherinopsidae) based on the mitochondrial ND2 gene. Copeia. (2):408–417. http://dx.doi.org/10.1643/CI-07-151

Bloom DD, Weir JT, Piller KR, Lovejoy NR. 2013. Do freshwater fishes diversify faster than marine fishes? A test using state-dependent diversification analyses and molecular phylogenetics of new world silversides (Atherinopsidae). Evolution. 67(7):2040–2057. http://dx.doi.org/10.1111/evo.12074

Charlier J, Palme A, Laikre L, Andersson J, Ryman N. 2011. Census (N*c*) and genetically effective (N*e*) population size in a lake-resident population of brown trout *Salmo trutta.* J Fish Biol. 79(7):2074–2082. http://dx.doi.org/10.1111/j.1095-8649.2011.03124.x

Clarke LM, Munch SB, Thorrold SR, Conover DO. 2010. High connectivity among locally adapted populations of a marine fish (*Menidia menidia*). Ecology. 91(12):3526–3537. http://dx.doi.org/10.1890/09-0548.1

Clement M, Posada DCKA, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. Mol Ecol. 9(10):1657–1659. http://dx.doi.org/10.1046/j.1365-294x.2000.01020.x

Conover DO. 1998. Local adaptation in marine fishes: evidence and implications for stock enhancement. Bull Mar Sci. 62(2):477–493.

Conover DO, Munch S, Lankford T Jr. 2000. Current status of the key silverside, *Menidia conchorum*, in southern Florida Final Project Report. US Geological Survey Biological Resources Division Restoration Ecology Branch, Florida Caribbean Science Center, Florida International University, Miami, FL 33199. 18 p.

Conover DO, Present TMC. 1990. Countergradient variation groth rate: Compensation for length of the growing season among Atlantic silversides from different latitudes. Oecologia. 83(3):316–324. http://dx.doi.org/10.1007/BF00317554

Cracraft J. 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. *In:* Otte D, Endler J, editors. Speciation and its consequences. Sunderland (MA): Sinauer. p. 28–59.

Duggins C Jr, Karline A, Relyea K, Yerger R. 1986. Systematics of the key silverside *Menidia conchorum* with comments on other Menidia species (Pisces Atherinidae). Tulane Stud Zool Bot. 25(2):133–150.

Echelle AA, Kornfield I. 1984. Evolution of fish species flocks. University of Maine Press.

Eschmeyer WN, Fricke R, Fong JD, Polack DA. 2010. Marine fish diversity: history of knowledge and discovery (Pisces). Zootaxa. 19–20.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Mol Ecol. 14(8):2611–2620. http://dx.doi.org/10.1111/j.1365-294X.2005.02553.x

Excoffier L, Lischer HE. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 10(3):564–567. http://dx.doi.org/10.1111/j.1755-0998.2010.02847.x

Fluker BJ, Pezold F, Minton RJ. 2011. Molecular and morphological divergence in the Inland silverside (*Menidia beryllina*) along a freshwater-estuarine interface. Environ Biol Fishes. 91(3):311–325. http://dx.doi.org/10.1007/s10641-011-9786-2

Frankham R. 1996. Relationship of genetic variation to population size in wildlife. Conserv Biol. 10(6):1500–1508. http://dx.doi.org/10.1046/j.1523-1739.1996.10061500.x

Frasier TR. 2008. STORM: software for testing hypotheses of relatedness and mating patterns. Mol Ecol Resour. 8(6):1263–1266. http://dx.doi.org/10.1111/j.1755-0998.2008.02358.x

Getter C. 1981. Ecology and survivial of the key silverside, *Menidia conchorum*, an atherinid fish endemic to the Florida Keys. PhD Dissertation. University of Miami.

Goudet J. 1995. FSTAT version 1.2: a computer program to calculate F statistics. J Hered. 86(6):485–486.

Hale ML, Burg TM, Steeves TE. 2012. Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PLoS ONE. 7(9):e45170. http://dx.doi.org/10.1371/journal.pone.0045170

Hildebrand S, Ginsburg I. 1927. Descriptions of two new species of fishes from Key West, Florida, with notes on nine other fishes collected in the same locality. Fish Bull Fish Wildl Serv. 42:207–215.

Hoffmeister JE, Multer HG. 1968. Geology and Origin of the Florida Keys. Geol Soc Am Bull. 79(11):1487–1502.http://dx.doi.org/10.1130/0016-7606(1968)79[1487:GAOOTF]2.0.CO;2

Hughes JM, Real KM, Marshall JC, Schmidt DJ. 2012. Extreme genetic structure in small-bodied freshwater fish, the purple spotted gudgeon, *Mogurnda adspersa* (Eleotridae). PLoS ONE. 7(7):e40546. http://dx.doi.org/10.1371/journal.pone.0040546

Klingenberg CP. 2011. MorphoJ: an integrated software package for geometric morphometrics. Mol Ecol Resour. 11(2):353–357. http://dx.doi.org/10.1111/j.1755-0998.2010.02924.x

Kubota H, Watanabe K, Suguro N, Tabe M, Umezawa K, Watanabe S. 2010. Genetic population structure and management units of the endangered Tokyo bitterling, *Tanakia tanago* (Cyprinidae). Conserv Genet. 11(6):2343–2355. http://dx.doi.org/10.1007/s10592-010-0120-x

Lynch M, Lande R. 1998. The critical effective size for a genetically secure population. Anim Conserv. 1(1):70–72. http://dx.doi.org/10.1111/j.1469-1795.1998.tb00229.x

May RM. 1994. Biological diversity: differences between land and sea. Oceanogr Lit Rev. 12(42):1094.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89(3):583–590.

Plank SM, Lowe CG, Feldheim KA, Wilson Jr. RR, Brusslan JA. 2010. Population genetic structure of the round stingray *Urobatis halleri* (Elasmobranchii: Rajiformes) in southern California and the Gulf of California. J Fish Biol 77:329–40. http://dx.doi.org/10.1111/j.1095-8649.2010.02677.x

Poulakis GR, Blewett DA, Mitchell ME. 2003. The effects of season and proximity to fringing mangroves on seagrass-associated fish communities in Charlotte Harbor, Florida. Gulf Mex Sci. 21:171–184.

Pritchard JK, Stephens M, Donelly P. 2000. Inference of population structure using multilocus genotype data. Genetics. 155(2):945–959.

Puebla O. 2009. Ecological speciation in marine v freshwater fishes. J Fish Biol. 75(5):960–996. http://dx.doi.org/10.1111/j.1095-8649.2009.02358.x

Raymond M, Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Heredity. 86(3):248–249.

Rohlf FJ. 2010. tpsDIG2 v2.16. http://life.bio.sunysb.edu/morph/. Accessed 17 February, 2013.

Rohlf FJ. 2011. tpsRegr v1.38. http://life.bio.sunysb.edu/morph/. Accessed 17 February, 2013.

Rousset F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Resour. 8(1):103–106. http://dx.doi.org/10.1111/j.1471-8286.2007.01931.x

Sanz N, Fernandez-Cebrian R, Casals F, Araguas RM, Garcia-Marin JL. 2011. Dispersal and demography of brown trout, *Salmo trutta*, inferred from population and family structure in unstable Mediterranean streams. Hydrobiologica. 671(1):105–119. http://dx.doi.org/10.1007/s10750-011-0708-2

Sbrocco EJ, Barber PH. 2011. Ten polymorphic microsatellite loci for the Atlantic silverside, *Menidia menidia.* Conserv Genet Resour. 3(3):585–587. http://dx.doi.org/10.1007/s12686-011-9410-y

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol. 18(2):233–234. http://dx.doi.org/10.1038/72708

Seymour M, Rasanen K, Holderegger R, Kristjansson BK. 2013. Connectivity in a pond system influences migration and genetic structure in threespine stickleback. Ecol Evol. 3(3):492–502. http://dx.doi.org/10.1002/ece3.476

Suk HY, Neff BD, Quach K, Morbey YE. 2012. Evolution of introduced Chinook salmon (*Oncorhynchus tshawytscha*) in Lake Huron: emergence of population genetic structure in less than 10 generations. Ecol Freshwat Fish. 21(2):235–244. http://dx.doi.org/10.1111/j.1600-0633.2011.00542.x

Thompson JD, Gibson TJ, Higgins D. 2002. Multiple sequence alignment using ClustalW and ClustalX. Current Protocols in Bioinformatics 2:1–22.

Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P. 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes. 4(3):535–538. http://dx.doi.org/10.1111/j.1471-8286.2004.00684.x

Waples RS. 2006. A bias correction for estimates of effective population size based on linkage disequilibrium at unlinge gene loci. Conserv Genet. 7(2):167–184. http://dx.doi.org/10.1007/s10592-005-9100-y

Waples RS, Do C. 2008. LDNe: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Resour. 8(4):753–756. http://dx.doi.org/10.1111/j.1755-0998.2007.02061.x

Ward RD, Woodwark M, Skibinski DOF. 1994. A comparison of genetic diversity levels in marine, freshwater, and anadramous fishes. J Fish Biol. 44(2):213–232. http://dx.doi.org/10.1111/j.1095-8649.1994.tb01200.x

Wilson EO, Brown WL Jr. 1953. The subspecies concept and its taxonomic application. Syst Zool. 2(3):97–111. http://dx.doi.org/10.2307/2411818

Yamahira K, Conover DO. 2002. Intra- vs interspecific latitudinal variation in growth: adaptation to temperature or seasonality? Ecol Evol. 83(5):1252–1262.

Yamahira K, Kawajiri M, Takeshi K, Irie T. 2007. Inter- and intrapopulation variation in thermal reaction norms for growth rate: evolution of latitudinal compensation in ecotherms with a genetic constraint. Evolution. 61(7):1577–1589. http://dx.doi.org/10.1111/j.1558-5646.2007.00130.x

Zelditch ML, Swiderski DL, Sheets HD, Fink WL. 2004. Geometric morphometrics for biologists: a primer. San Diego: Elsevier Academic Press. 443 p.