Genetic Population Structure and Accuracy of Morphological Assessment in *Alosa aestivalis* (Blueback Herring) and *A. pseudoharengus* (Alewife)

Christopher Kan[1,*], John Lichter[2], and Vladimir Douhovnikoff[2]

Abstract - *Alosa aestivalis* and *A. pseudoharengus* are herring congeners that are important forage species for piscivorous fish and birds. We measured population structure metrics for these species using microsatellite markers. The Southern Gulf of Maine study area allowed the assessment of these species at an inter- and intra-watershed level. We found no detectable population structure within or among watershed for either species which agrees with other recent research. Our results support regional-scale (e.g., Gulf of Maine) plans for management for *A. aestivalis* and *A. pseudoharengus*. We found that 5.4% of our samples were hybrids. Our study adds to a growing body of evidence that hybridization and introgression should be management concerns for these species, and precautions should be taken to preserve species barriers. An error rate of morphological identification was calculated by comparing morphological identifications against genetic classifications. We found an overall identification error rate of 16%, which differed significantly from zero ($P = 0.008$). Managers should also take note of the uncertainty in morphological identifications and adjust stock models and policies accordingly.

Introduction

Alosa aestivalis (Mitchell) (Blueback Herring) and *A. pseudoharengus* (Wilson) (Alewife) are in the sub-genus *Pomolobus,* a grouping that has been supported strongly by recent phylogenetic studies (Bowen et al. 2008, Faria et al. 2006), and pomoloboids is our preferred label for these 2 species, rather than the now paraphyletic designation river herring that includes *Alosa sapidissima* (Wilson) (American Shad). Pomoloboids are anadromous and co-occur for much of their range from northern Florida to the Gulf of Saint Lawerence (NOAA 2013). They are an ecologically important link between riparian and marine ecosystems because their spawning material and carcasses support microbial and invertebrate populations through increased nutrient availability (Durbin et al. 1979). The seaward migration of young-of-the-year (YoY) fish reciprocally transports nutrients from freshwater to marine environments (Limburg and Waldman 2009), enhancing the spawning grounds for commercially important fish such as *Gadus morhua* (L.) (Atlantic Cod; Ames 2004) and for endangered *Salmo salar* (L.) (Atlantic Salmon; Saunders et al. 2006). However, pomoloboids have declined across much of their range, leading to their recent consideration for listing under the Endangered Species Act (National

[1]Ecology and Evolutionary Biology, University of California Santa Cruz, Santa Cruz, CA 95064. [2]Biology Department, Bowdoin College, Brunswick, ME 04011. [*] Corresponding author - chkan@ucsc.edu.

Manuscript Editor: Trevor Avery

Oceanic and Atmospheric Administration 2013). Deforestation and dams have driven local loss in habitat and, in conjunction with overfishing and pollution, have devastated stocks (Lichter et al. 2006). The collapse of populations, combined with the recognition of their ecological importance, has spurred restoration efforts.

Restoration efforts such as dam removals and water-quality improvement measures have recently shown signs of success with a robust recovery of pomoloboids in several Maine rivers (ASMFC 2012). Continued, enhanced, and broadened efforts are required to increase recovery of these species. A fuller understanding of the genetic population structure would help better target restoration efforts and allow management to operate at the appropriate spatial scale (Weston et al. 2016), but conflicting reports exist over the level of genetic population structure in North American pomoloboids at the inter- and intra-watershed levels (Hasselman et al. 2010, Waters et al. 2000). Watershed-level analyses are necessary because fish move within and between freshwater bodies, lakes, and rivers, using different oceanic outlets. Assessing the accuracy of morphological identification methods would also benefit managers who rely on morphological identifications for stock assessments and modeling. Knowledge of the uncertainty of the identifications is a key variable that affects model accuracy. Therefore, a better understanding of both the population structure of and the accuracy of morphological identification of pomoloboids would benefit restoration and management of the species.

This study seeks to help (1) address whether pomoloboids show population structure at the intra- and inter-watershed levels and (2) ascertain the accuracy of morphological species identification of pomoloboids. Both aims will be addressed through a microsatellite analysis (Julian and Bartron 2007). Results from this study will help to address knowledge gaps in the study area, and help to inform current management practices such as deciding appropriate spatial scales for restoration and including reliable indicators of morphological identification.

Materials and Methods

Field sites

Our 8 field sites were in estuaries and rivers draining into the Gulf of Maine (Fig. 1). Most sample sites (5 of 8) were within the Merrymeeting Bay watershed, which drains 6 rivers comprising a quarter of Maine's geographic area and part of New Hampshire (24,755 km^2). The bay served as the focal point of this study because its complex hydrology and multiple branches consisting of numerous ponds, lakes, and rivers allowed the investigation of intra-watershed population structure. The Kennebec and Androscoggin Rivers are the major rivers in the watershed (Fig. 1). The bay, which is estuarine and has a typical salinity of <5 but is tidally influenced and can have elevated salinity during periods of low river flow, has tidal marshes and sand flats which provide important habitat for birds (Kistner and Pettigrew 2001, Wong and Townsend 1999). Most samples were taken from freshwater rivers or lakes feeding into the bay.

All sample locations were in freshwater except the Oyster River site and Winnigance Lake, which can be brackish depending on flow and tidal conditions. All

river and lakes in the study except Winnigance Lake had dams and fish ladders associated with their outlets. The sample sites have generally been affected by pollution and overfishing in the past (Köster et al. 2007). Each site has one or more man-made structures, such as dams on the Androscoggin River or power plants on the Kennebec River, or settlements upstream that influence flow and ecology (Stewart et al. 2006).

Sample collection and field identification

We collected 184 samples in total, of which 106 were adults and 78 were juveniles (Table 1). Our collections included both adult *A. aestivalis* and juvenile samples that we identified to the genus level *Alosa,* meaning they could be 1 of 3 species: *A. aestivalis*, *A. pseudoharengus,* or *A. sapidissima*. Adults and juveniles were caught and identified using different means. We collected adult *A. aestivalis* with fyke nets and coastal seining between 3 June 2012 and 25 April 2012, and the specimens were identified by experts from Dr. Theodore Willis's lab at the

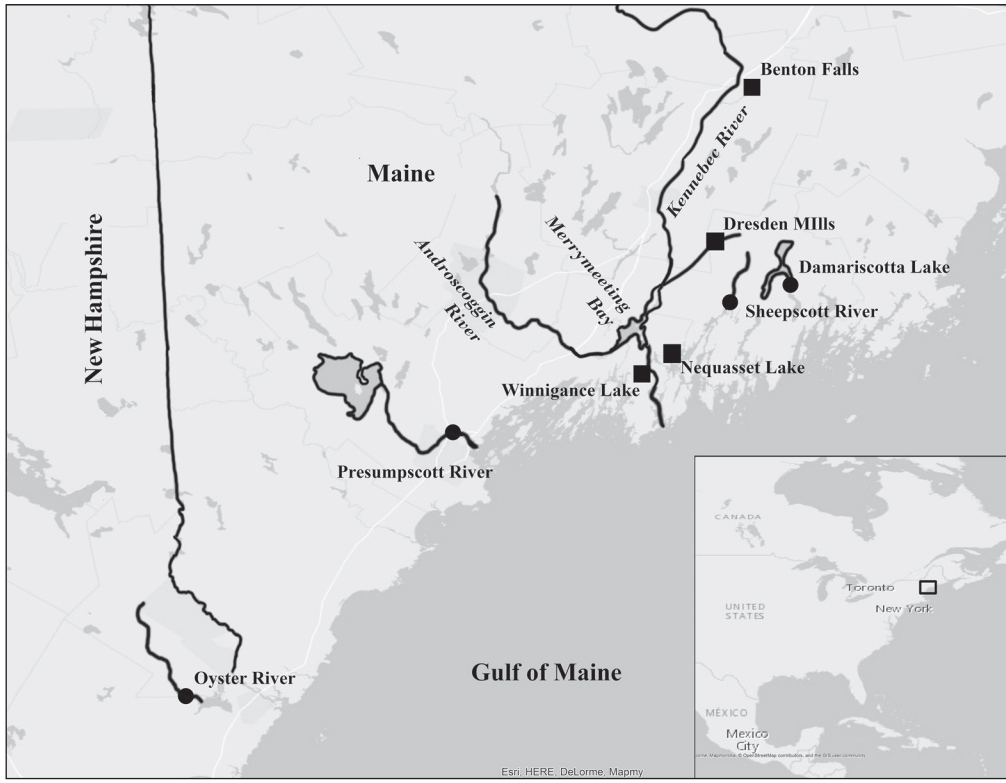

Figure 1. Map of samples sites, where *A. aestivalis* and *A. pseudoharengus*, from Southern New Hampshire to Midcoast Maine were collected. Samples were collected with fyke nets or beach seines in rivers and estuaries between June and August 2012. Samples sites in the Merrymeeting Bay watershed are shown with a black square. Sample sites in other watersheds are indicted with a black dot. Watersheds included in this study are highlighted in bold non-italicized text. Italicized text indicate geographic features other than sample sites and are for geographic reference only.

Table 1. Summary of collections made, showing sample sites, what types of samples were caught, the date(s) that they were visited, and what species, *A. pseudoharengus* (Ale), *A. aestivalis* (BB), or hybrid, were caught based on a classification by a genetic simulation protocol.

Site	Dates caught	Sample type	# of BB	# of Ale	# of hybrids	Total #	% BB	% Ale	% hybrid
Dresden Mills	1 June 2012	Adult	29	1	2	32	90.60%	3.13%	6.25%
Benton Falls	13 June 2012	Adult	23	2	2	27	85.20%	7.41%	7.41%
Nequasset Dam	9 August 2012	Juvenile	0	18	0	18	0.00%	100.00%	0.00%
Sheepscot River	8 July 2012, 12 July 2012	Juvenile	2	21	0	23	8.70%	91.30%	0.00%
Presumpscot River	1 August 2012	Juvenile	0	14	0	14	0.00%	100.00%	0.00%
Damriscotta River	13 August 2012	Juvenile	0	23	0	23	0.00%	100.00%	0.00%
Winnigance Lake	12 June 2012	Adult	21	0	0	21	100.00%	0.00%	0.00%
Oyster River	2 July 2012	Adult	16	4	6	26	61.50%	15.40%	23.10%
		Total	91	83	10	184	49.50%	45.10%	5.43%

University of Southern Maine. The identification used the color and luminosity of the peritoneum, light and shiny in *A. pseudoharengus* and dark and dusky with spots in *A. aestivalis*. This is the standard method for distinguishing between *A. aestivalis* and *A. pseudoharengus* . We were not able, due to logistical constraints, to target rivers in which adult *A. pseudoharengus* predominated; therefore, adult samples sizes of this species were low. We collected juvenile samples of this species with a 50-foot beach seine between 1 June 2012 and 13 August 2012. Sampling information including sampling date are provided in Table 1. We identified juvenile samples to the genus *Alosa* using body shape, head shape, and mouth morphology (Leim and Scott 1966).

We used only adult samples to test the accuracy of the peritoneum identifications. It is very difficult to reliably identify juveniles through morphology to species level (MacLellan et al. 1981); thus, they were not used to test morphological species identifications. All samples were used to investigate population structure in pomoloboids.

DNA extraction protocol, PCR amplification, and genotyping

We utilized 7 microsatellite markers that were originally developed in *A. sapidissima* for the genus *Alosa* to characterize the samples in the study (Table 2; Julian and Bartron 2007). Phenol-chloroform extractions were performed based on a protocol by Taggart et al. (1992). Briefly, we placed ~150 mg of skeletal muscle tissue in a 2.5-ml tube, and added 600 µl of CTAB Mixture, consisting of 0.2 M EDTA (ethylenediamine-tetraacetic acid), 2% Cetyl trimethylammonium bromide, and 25 µl of protinase K solution 20 mg/ml, and incubated the sample for 8 hours at 37 °C. Following incubation, we added 10 µl of 2 mg/ml RNAase A, DNAse and protease-free solution to the tube and mixed it. The sample was then incubated for 1 hour at 37 °C. After this, we aded 600 µl of Phenol-Chloroform-Isoamyl Alcohol (PCI) in a 24:24:1 ratio to each tube. Samples were mixed and centrifuged to separate layers. The bottom PCI layer was then removed, 600 µl of Chloroform-Isoamyl in a 24:1 ratio (CI) was added, and the tube was mixed and centrifuged again. The top aqueous layer containing DNA was removed to a new tube using a wide bore pipet. We repeated this PCI–CI process once more for a cleaner sample because we found that doing so resulted in better DNA amplification for our samples.

We then added isopropyl alcohol (600 µl) and 60 µl of 10.5 M Ammonium Acetate to the isolated DNA and decanted the solution from the precipitated DNA after centrifuging. Thereafter, 600 µl of -20°C 99% ethanol was added to the tube, centrifuged, and then decanted. We dried the tubes for 10 mins at room temperature and then thoroughly washed them with 50 µl of Tris-EDTA (10mM Tris, 1mM EDTA; ph 8.0) and allowed them to sit for 24 hours to achieve full dissolution.

We performed PCR amplification for all primers with either a standard or touchdown protocol (Table 2). The standard protocol consisted of a hot start with (1) an initial denaturing at 94 °C for 2 min, (2) denaturing at 94 °C for 45 s, (3) annealing at 56 °C for 45 s, (4) extension at 72 °C for 2 min, and (5) final extension at 72 °C for 5 min. We repeated steps 2–4 for 35 cycles. The touchdown protocol involved

Table 2. Information about the primers used and resulting amplicons for *A. pseudoharengus* (Ale) and *A. aestivalis* (BB). All primers used the FAM fluorophore except AsaD042, which used MAX (VIC substitute). Primers were originally from Julian and Bartron (2007).

Primer	Genbank accession no.	Repeat motif	PCR amplification conditions	Primer sequences (5'–3')	No. of alleles (Ale)	No. of alleles (BB)	Ale size (max–min bp)	BB size (max–min bp)
AsaC010	EF014991	$(GTAT)_{16}$	Touchdown	F: AATAATGTTGTGCTGGATTGTG R: TTTATTGTTATTGTGATGGAGGG	7	7	264–308	264–308
AsaC249	EF014994	$(CATA)_8$ $(TTCT)_{13}$	Standard	F: TTATTACAACGGTGAATTGAGTG R: TAAGTGCATGTTGTGTGTGATG	7	2	193–221	193–221
AsaD021	EF014996	$(CTAT)_{15}$	Standard	F: CTCTCTTCCCCATCACTCTTC R: CAAAGCCCTCGTTTAGTTATTC	5	6	260–276	216–284
AsaD029	EF014997	$(CTAT)_{20}$	Touchdown	F: ATTATGCACAGGAATCTGGAAG R: TGTGCTTACAAAAGTGACATGG	9	16	216–284	216–284
AsaD030	EF014998	$(CTAT)_{23}$	Touchdown	F: CCACAGCATCATCTCTTTACTG R: ACCTTGAATTTCTCCTTGGG	6	9	84–122	84–122
AsaD042*	EF015000	$(CTAT)_{12}$	Touchdown	F: ACTGGTCAATTGTAAGACACCC R: CAAGATGACCAAGGGTTAAGAC	7	10	144–202	144–202
AsaD055	EF015001	$(CTAT)_{10}$	Touchdown	F: CTCTTTCACAGGGATCAAAGTC R: CAAGCATGTTTAAATAGGAGGC	6	12	247–312	247–213

a starting annealing temperature of 64 °C, which was reduced by 0.5 °C each additional cycle until, at cycle 10, a final annealing temperature of 54 °C was reached. This final temperature was used for annealing in cycles 11–35. The other steps and parameters in the touchdown protocol were the same as the standard protocol.

Microsatellites were genotyped on an ABI 3730xL DNA Analyzer at the Harvard Biopolymers Facility (Cambridge, MA) and were scored using Applied Bio Systems PeakScanner software 2.0 (Carlsbad, CA). The allele calls were double checked by the initial researcher, and a random sample was triple checked for accuracy by other researchers. No significant deviation between researchers was found in allele calling. The data was checked in Microchecker v. 2.23 (Van Oosterhout et al. 2004) for genotyping and amplification errors such as allelic dropouts, excess small-allele amplification, and stutter products.

Genetic sample classification using a simulation-based protocol

We analyzed the microsatellite data to differentiate samples by species according to the protocol by Hasselman et al. (2014). Briefly, the protocol can be broken into 2 basic steps: (1) perform computer hybridization simulations to determine which q-values (a numerical value indicating a sample's likely genetic makeup and ancestry) correspond to purebred or hybrid individuals, and (2) run programs on collected samples to obtain q-values and use the benchmarks from simulations to identify species. The meaning of a q-value can change based on the species studied and the markers used (Evanno et al. 2005, Hasselman et al. 2014). Hence simulations of hybridization and backcrosses of assumed purebreds are a best practice for determining the likely ancestry of an individual for any given q-value.

We ran the 154 most pure individuals, defined as having a q score ≥0.9 by STRUCTURE v2.3.4 (Pritchard et al. 2000), through simulated hybridization and backcross events in Hybrid Lab v1.0 (Nielsenet al. 2006). Five simulated data sets resulted from this exercise. The first step we took was using Hybrid Lab to create 200 genetic profiles for each species. These 400 purebred parental individuals were then used to generate 100 F1 hybrids, which in turn were used to generate 100 F2 hybrids and 100 F1 hybrids backcrossed with purebred parents, hereafter referred to as F1 backcrosses. We analyzed each of these simulated groups in STRUCTURE and New Hybrids v1.1 (Anderson and Thompson 2002) to determine which ancestry scores, q-values in STRUCTURE and PofZ in New Hybrids, corresponded to different levels of introgression. This approach allowed us to have benchmarks for each software for what constituted a purebred or a hybrid individual. It also allowed us to assess what each program was capable of discerning. For example, whether first generation hybrids could be distinguished from F1 backcrosses.

Each simulated dataset of hybrids was run in STRUCTURE with the admixture model for 100k burn-in steps, 500k iterations and, 5 repetitions for each K (the number of genetic clusters, 1 thru 6). Results were combined across repetitions using Structure Harvester (Earl and vonHoldt 2012) and CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and visualized with distruct v1.1 (Rosenberg 2004) or Structure Plot (Ramasamy et al. 2014). We identified the ideal K per the

Evanno method (Evanno et al. 2005). The Evanno method expands on the original Prichard paper, which suggested using Ln(K), also referred to as L(K), to find the true value of K, and proposes using instead delta K for times when the real value of K is greater than 1. The delta K metric was found to be better than Ln(K) at detecting the true K through simulations for Ks > 1 (Evanno et al. 2005, Pritchard et al. 2000). Using this information from these methods, we assessed for each q-value the likelihood and confidence of species classification. For a q-score in an analysis with only 2 clusters, 0 is one purebred species and 1 is the other. Hence all individuals are more like one cluster or the other depending on the score. For example, in our study, if 1 is strongly *A. pseudoharengus* and 0 is *A. aestivalis*, a q-value of 0.8 in STRUCTURE might mean a sample has a 90% change of being an *A. pseudoharengus*, 9% of being a *A. aestivalis* and 1% of being a hybrid. We found during our testing that STRUCTURE was not able to distinguish between different types of hybrids, but was easily able to distinguish between the 2 purebred species and between purebreds and hybrids.

New Hybrids was run for 10k burn-in steps. 200k iterations, and replicated 4 times per simulated dataset. The program outputs a PofZ value for each sample that gives a percent chance a given sample is a member of 6 possible classes: purebred species 1 or 2, F1 hybrid, F2 hybrid, or a F1 backcross with species 1 or species 2. We found in our simulations that New Hybrids was not capable of distinguishing between the hybrid categories within our data, likely due to the number of markers. We therefore summed all the hybrid categories into a single hybrid category that resulted in a sufficient signal to distinguish between sample types. Hence, we were only asking New Hybrids to distinguish between 3 types of samples: pure *A. aestivalis*, pure *A. pseudoharengus* or hybrids of any of the 4 aforementioned types.

This group structure allowed us to convert these PofZ numbers to a q-value equivalent since New Hybrids was easily able to distinguish between pure *A. aestivalis* and pure *A. pseudoharengus* in our simulations, i.e., if one PofZ probability was high for one of the parent species the PofZ probability for the other would be near zero. New Hybrids easily distinguished between the simulated purebred samples but occasionally had difficulty distinguishing purebreds and hybrids. Using these principals, we converted a PofZ to a q-value like number as follows. For example, where a PofZ indicates the chance of *A. aestivalis* as 90%, *A. pseudoharengus* as <1%, and the hybrid as 10%, New Hybrids treats the sample as *A. aestivalis* with the small chance of it being a hybrid. If we think back to the q-value scale in the case of K = 2, where *A. aestivalis* as 0 and *A. pseudoharengus* as 1, we can convert the PofZ to a q-value of 0.10. One can think of the hybrid percentage as the deviation from the purebred percentage; hence, it can be added in the case of *A. aestivalis* to 0 or subtracted for *A. pseudoharengus* from 1. Thus, looking at another example, a PofZ of *A. aestivalis* as <1%, *A. pseudoharengus* as 70%, and the hybrid as 30% would be 1 - 0.3 = 0.7 = q-value.

We used a simple species identification consensus score for each of our 184 samples based on the information obtained from our hybridization simulations with STRUCTURE and New Hybrids. For a sample of one of our simulations, see

Supplemental Table 1 (available online at https://www.eaglehill.us/NENAonline/suppl-files/n24-4-N1442-Kan-s1, and, for BioOne subscribers, at https://dx.doi.org/10.1656/N1442.s1). The synthetic consensus score was from 0 to 2, where scores of 0, 1, or 2 were possible. Zero meant a disagreement of both programs binning the sample into the 2 parent species, the greatest possible level of disagreement; this score never occurred in our samples. One meant one program identified a sample as a hybrid and the other identified it as a purebred. Two meant that both programs identified a sample as a hybrid or as one of the parent species. The samples that scored 1 were manually reviewed by researchers and final classification judgments were made using the statistical information obtained from the Hybrid Lab simulations.

Analyzing each species for population structure and other metrics

We analyzed samples identified as *A. aestivalis* and *A. pseudoharengus* for population structure in separate species-specific STRUCTURE and New Hybrids runs using the same burn-in steps, Ks, iterations, and repetitions parameters as above. The Evanno method (Evanno et al. 2005) was applied again to find the most likely K and number of genetic clusters in the sample to infer genetic structure at the inter- and intra-watershed scale. We also used identifications from the STRUCTURE and New Hybrids analyses to determine the accuracy of morphological field identifications of adult samples. Deviation from the null hypothesis of no identification error was evaluated using Fisher's Exact Test in JMP Pro 11 (Cary, NC). We analyzsed species data sets in Genepop v4.2 (Rousset 2008) to calculate tests for Hardy Weinburg Equlibrium and to calculate expected and observed heterozygosity, allelic richness, and F_{IS}. G-statistic based *P*-values were calculated in FSTAT v. 2.9.3.2 (Goudet 1995). Statistical tests underwent sequential Bonferroni correction (Holm 1979) to account for the effects of multiple statistical tests. The Holm method is more powerful than Bonferroni correction (Allendorf and Phelps 1981). We categorized private alleles, alleles exclusive to one species, using GenALEx v6.5 (Peakall and Smouse 2012).

Results

Of 184 samples, none scored 0 on our species identification score, 6 scored 1, and 178 scored 2. Thus, for 97% of our samples, STRUCTURE and New Hybrids agreed on individual identification as hybrid, *A. aestivalis,* or *A. pseudoharengus* (Table 1). This result suggests the analysis could easily classify most samples. The analysis did not indicate the presence of a third species in our sample (Figure 2Ai–Aiii). This result means that no *A. sapidissima,* the third alosid present in the system, were found during sampling. The markers used had greater or different bp size ranges from those originally reported for *A. pseudoharengus* and *A. aestivalis* in Julian and Bartron (2007). We believe that we found different bp size ranges because we used larger sample sizes of 91 and 83 per species rather than 2–3 per species as in Julian and Bartron (2007). Some of the size variation is also likely due to regional variation in genetic composition since our samples were from the Gulf of Maine and those in Julian and Bartron (2007) were from Long Island Sound. The

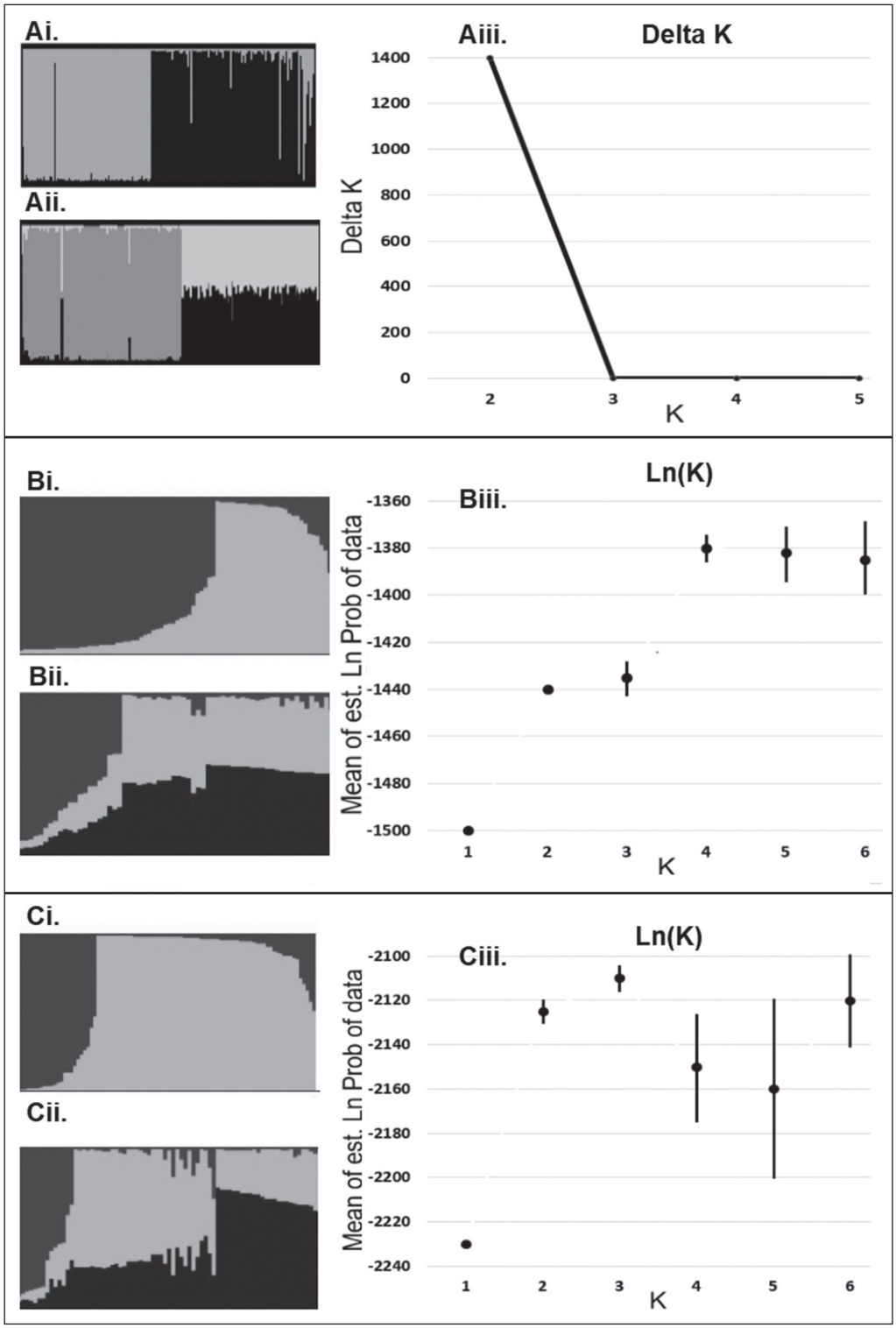

Figure 2. [Caption on following page.]

size ranges for both species in our sampling are identical (Table 2), which is not surprising due to hybridization between the species facilitating allele exchange.

A *t*-test found a significant departure ($P = 0.008$) from the null hypothesis that the morphological identifications had no significant difference from the genetic classifications. Of the 106 morphologically identified adult *A. aestivalis* that we sequenced, 7 were later genetically classified as *A. pseudoharengus* and 10 as hybrids. We calculated 2 error rates, one for errors where adult *A. pseudoharengus* were mistaken as *A. aestivalis* and a second for those that resulted from hybrid adults, that were likely morphologically cryptic. These rates are 6.6% and 9.4% respectively, for an overall morphological identification error rate of 16.0%.

We found no evidence for genetic population structure at inter- or intra-watershed levels for either species. Admixture coefficient plots generated by STRUCTURE (Fig. 2B, C) show 2 potential intra-species clusters within *A. aestivalis,* and *A. pseudoharengus* (Fig. 2Bi–ii , Ci–ii), but we determined that both species likely only had a single cluster because of the low and non-significant F_{ST}s between the potential clusters. The F_{ST}s were 0.01 ($P = 0.67$) and 0.002 ($P = 0.55$) for *A. pseudoharengus* and *A. aestivalis*, respectively. Since the Evanno method cannot be used to detect single clusters of K = 1 (Evanno et al. 2005), log probability plots, Ln(K), were used instead (Fig. 2Biii, Ciii). The plots support the hypothesis that the most likely number of clusters is 1, suggesting there is no detectable genetic structure within either species in our sample. Most markers showed significant divergences from the Hardy–Weinberg equilibrium (HWE) in both *A. aestivalis* and *A. pseudoharengus* (Table 3). The percentage of hybrids across sites was low, comprising 5.43% of all samples. The Oyster River, which has the slowest and narrowest river area, had the highest percentage of hybrids (Table 1). Limited sample sizes (Table 1) per site likely prevented us from finding any relationship between sample site and the number of hybrids ($P = 0.310$).

Discussion

Our results support no genetic population structure at the intra- or inter-watershed level within our Gulf of Maine study region. Our study adds to a growing line of evidence from studies covering the eastern seaboard that genetic structure for pomoloboids is best discernable at region-wide scales like the Gulf of Maine rather than at watershed scales (McBride et al. 2014, Palkovacs et al. 2014). Results suggest 7 markers were sufficient for this analysis due to the strong performance

Figure 2. STRUCTURE, Evanno method, and log probability plots to identify the correct number of genetic clusters. Panel A shows the STRUCTURE and Evanno plots for a combined sample of *A. pseudoharengus* and *A. aestivalis* at K = 2 (Ai.) and K = 3 (Aii.) (F_{ST} = 0.227). The subsequent 2 panels for (B) *A. pseudoharengus* and (C) *A. aestivalis* show STRUCTURE plots at K = 2 and K = 3 with 2 clusters evident. However, low F_{ST} values (0.017 and 0.002, respectively) indicate that there is only 1 true cluster in both species. The Evanno method cannot detect only 1 cluster, so a graph of the recommended alternative method using the log probability of the data is shown in the right column as Biii and Ciii. All STRUCTURE plots are K = 2 on top and K = 3.

that New Hybrids and STRUCTURE exhibited in simulations when distinguishing between purebreds and hybrids. We caution though that STRUCTURE and New Hybrids results should be analyzed in the context of significant deviations from HWE in most markers, which can affect the results of both models (Table 3). The deviations could be a result of introgression. The evidence from our study and others suggests that the Gulf of Maine is likely a genetic unit that has significant internal gene flow and that regional-scale management for fisheries and conservation may be appropriate for pomoloboids.

Our results along with the recent genetic studies by Hasselman et al. (2014) and Mcbride et al. (2014) suggest that pomoloboids have a clear potential for hybridization. Our study found 5.4% of our samples were hybrids; however, it was not feasible to determine the precise type of hybrid due to insufficient statistical power. Our finding that the size ranges of the microsatellite alleles for these 2 species exactly overlap suggests that introgression has allowed the reciprocal movement of alleles overtime. Our findings are supported by Hasselman et al. (2014), which documented the formation of a hybrid swarm in a lake after a dam was built, and Mcbride et al. (2014), which documented F1 hybrids and advanced backcrosses. These studies suggest there are many opportunities for hybridization since the species overlap over much of their range and during much of their spawning time in freshwater. When the 2 species were found to co-occur, both Hasselman et al. (2014) and Mcbride et al. (2014) found increased levels of hybridization These findings are consistent with our observations of hybridization. Even though our sampling approach at each site did not always capture both species, we believe that this is further evidence that they can and do interbreed and that the chance of hybridization increases with the level of interspecies contact.

Genetic and ecological evidence indicate that hybridization and the subsequent introgression of alleles is occurring between the 2 species. Therefore, managers should take caution to prevent the formation of hybrid swarms. It is probable that hybrids have always occurred between these species, but elevated numbers of hybrids backcrossing into parent species can erode the barriers to gene flow over time leading to the merging of gene pools. Activities such as habitat modification

Table 3. Population structure measures for *A. pseudoharengus* (Ale) and *A. aestivalis* (BB). Ho and He are observed and expected heterozygosity, respectively. *P*-values are *g*-statistic based for Hardy-Weinburg equilibrium (HWE). * indicates significant deviations from HWE of $P < 0.05$.

Primer	Ho (Ale)	He (Ale)	Ho (BB)	He (BB)	Ale *F*is	BB *F*is	Ale *P*-Value (HWE)	BB *P*-value (HWE)	Ale Allelic richness	BB Allelic richness
AsaC010	0.349	0.241	0.582	0.409	-0.461	-0.429	<0.001*	<0.001*	1.900	4.114
AsaC249	0.337	0.272	0.241	0.139	-0.243	-0.756	0.431	<0.001*	2.044	1.934
AsaD021	0.542	0.387	0.373	0.265	-0.407	-0.414	<0.001*	<0.001*	2.371	4.689
AsaD029	0.108	0.097	0.659	0.602	-0.116	-0.095	0.870	0.095	3.213	8.012
AsaD030	0.590	0.439	0.659	0.532	-0.347	-0.324	<0.001*	<0.001*	2.613	4.955
AsaD042	0.602	0.476	0.373	0.311	-0.267	-0.201	0.030*	0.012*	2.841	5.137
AsaD055	0.156	0.111	0.615	0.527	-0.424	-0.168	<0.001*	0.646	2.749	6.258

by dam building or bank engineering should be undertaken with caution, especially when they may reduce spawning space or cause crowding. Similarly, stocking could cause crowding by reducing the spawning space available per individual, or might introduce a new species to a watershed. Both habitat modification and stocking may lead the species to hybridize at a higher rate than historically found in the system since it will restrict their ability to segregate activities by species. For instance, Hasselman et al. (2014) documented that the construction of a dam caused 2 pomoloboids populations to collapse. The habitat modification caused by the dam perturbed the populations and they merged into a hybrid swarm. Similarly, stocking may increase straying compared to native individuals such as in salmonids (Quinn 1993, Stabell 1984). Therefore, managers must carefully consider events or actions, direct or indirect, that could affect the ability for the species to segregate when mating.

Hybridization could complicate stock monitoring and reduce the accuracy of the morphological identifications managers typically rely on for pomoloboids. We found that morphological identifications by experts using the peritoneum, the most common morphological identifier to distinguish between the 2 species (Leim and Scott 1966, MacLellan et al. 1981), had an error rate of 16%. This rate, considered adequate by the Food and Agriculture Organization of the UN (Fischer 2013:27), could still complicate or influence model predictions. Pomoloboids are notoriously difficult to identify by any morphological means due to their physical similarity. Yet, morphological methods, particularly the peritoneum, are still used as the primary identification method for stock assessments. Managers should note the level of error associated with these methods and incorporate it into the models used to determine stock policies such as management unit size and bycatch estimates.

Similar with other studies, genetic makeup may have been influenced by the history of extensive stocking of *A. pseudoharengus* in Maine (Hasselman et al. 2013) and recent recoveries (ASMFC 2012). It is possible that there were greater levels of genetic structure prior to human intervention and that stocking has reduced genetic population structure by homogenizing previously distinct populations (McBride et al. 2015). The effects of stocking are thought to be varied in pomoloboids depending on the level of selection in the populations (Bentzen et al. 2011).

Future work is still needed to clarify the parameters of alosine migration in recovering areas. Messieh (1977), using multivariate analysis of morphological characteristics, and Jessop (1994), using genetic markers, measured fidelity to natal rivers varying from 63% to 97%. It would be informative to know if fidelity to natal rivers changes with habitat degradation or recovery. For example, lower fidelity during recovery would facilitate re-colonization of newly available habitat. The measurement of this parameter would assist in models of population restoration or colonization that are critical to the conservation of *A. aestivalis* and *A. pseudoharengus*. Additional work regarding the frequency and mechanism of hybridization within pomoloboids would greatly inform restoration and efforts to preserve the genetic integrity of this taxon.

Acknowledgments

The authors thank their generous funders of this project: the Rusack Coastal Studies Fellowship, the Grua/o'Connell Research Award, the Roberts Fund, the Howard Hughes Medical Institute Fellowship, and Bowdoin Biology. We are also grateful to Theodore Willis for his assistance with analysis and samples. Finally, thank you to Daniel Hasselman, Nate Grey, Gail Wippelhauser ,and Mark Lazzari for their invaluable help with data collection and analysis.

Literature Cited

Allendorf, F.W., and S.R. Phelps. 1981. Use of allelic frequencies to describe population structure. Canadian Journal of Fisheries and Aquatic Sciences 38:1507–1514.

Ames, E.P. 2004. Atlantic cod stock structure in the Gulf of Maine. Fisheries 29:10–28.

Anderson, E.C., and E.A. Thompson. 2002. A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217–1229.

Atlantic States Marine Fisheries Commission (ASMFC). 2012. River Herring Benchmark Stock Assessment. Volumes I and II. Arlington, VA.

Bentzen, P., I. Paterson, M. McBride, and T.V. Willis. 2011. Genetic analysis of anadromous Alewife populations in Maine. Maine Rivers, Yarmouth, ME.

Bowen, B.R., B.R. Kreiser, P.F. Mickle, J.F. Schaefer, and S.B. Adams. 2008. Phylogenetic relationships among North American *Alosa* species (Clupeidae). Journal of Fish Biology 72:1188–1201.

Durbin, A.G., S.W. Nixon, and C.A. Oviatt. 1979. Effects of the spawning migration of the Alewife, *Alosa Pseudoharengus*, on Freshwater Ecosystems. Ecology 60:8–17.

Earl, D.A., and B.M. vonHoldt. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software structure: A simulation study. Molecular Ecology 14:2611–2620.

Faria, R., S. Weiss, and P. Alexandrino. 2006. A molecular phylogenetic perspective on the evolutionary history of *Alosa* spp. (Clupeidae). Molecular Phylogenetics and Evolution 40:298–304.

Fischer, J. (Ed.). 2013. Fish identification tools for biodiversity and fisheries assessments: Review and guidance for decision-makers. Food and Agriculture Organization of the United Nations, Rome, Italy.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate *F*-statistics. Journal of Heredity 86:485–486.

Hasselman, D.J., R.G. Bradford, and P. Bentzen. 2010. Taking stock: Defining populations of American Shad (*Alosa sapidissima*) in Canada using neutral genetic markers. Canadian Journal of Fisheries and Aquatic Sciences 67:1021–1039.

Hasselman, D.J., D. Ricard, and P. Bentzen. 2013. Genetic diversity and differentiation in a wide-ranging anadromous fish, American Shad (*Alosa sapidissima*), is correlated with latitude. Molecular Ecology 22:1558–1573.

Hasselman, D.J., E.E. Argo, M.C. McBride, P. Bentzen, T.F. Schultz, A.A. Perez-Umphrey, and E.P. Palkovacs. 2014. Human disturbance causes the formation of a hybrid swarm between two naturally sympatric fish species. Molecular Ecology 23:1137–1152.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6:65–70.

Jakobsson, M., and N.A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806.

Jessop, B.M. 1994. Homing of Alewives (*Alosa pseudoharengus*) and Blueback Herring (*A. aestivalis*) to and within the Saint John River, New Brunswick, as indicated by tagging data. Department of Fisheries and Oceans Canada, Biological Sciences Branch, Freshwater and Anadromous Division, Halifax, NS, Canada.

Julian, S.E., and M.L. Bartron. 2007. Microsatellite DNA markers for American Shad (*Alosa sapidissima*) and cross-species amplification within the family Clupeidae. Molecular Ecology Notes 7:805–807.

Kistner, D.A., and N.R. Pettigrew. 2001. A variable turbidity maximum in the Kennebec estuary, Maine. Estuaries 24:680–687.

Köster, D., J. Lichter, P.D. Lea, and A. Nurse. 2007. Historical eutrophication in a river–estuary complex in mid-coast maine. Ecological Applications 17:765–778.

Leim, A.H., and W.B. Scott. 1966. Fishes of the Atlantic coast of Canada. Bulletin of the Fisheries Research Board of Canada 155. Ottawa, ON, Canada.

Lichter, J., H. Caron, T.S. Pasakarnis, S.L. Rodgers, T.S. Squiers, and C.S. Todd. 2006. The ecological collapse and partial recovery of a freshwater tidal ecosystem. Northeastern Naturalist 13:153–178.

Limburg, K.E., and J.R. Waldman. 2009. Dramatic declines in North Atlantic diadromous fishes. BioScience 59:955–965.

MacLellan, P., G.E. (Buck) Newsome, and P.A. Dill. 1981. Discrimination by External Features between Alewife (Alosa pseudoharengus) and Blueback Herring (A. aestivalis). Canadian Journal of Fisheries and Aquatic Sciences 38:544–546.

McBride, M.C., T.V. Willis, R.G. Bradford, and P. Bentzen. 2014. Genetic diversity and structure of two hybridizing anadromous fishes (*Alosa pseudoharengus*, *Alosa aestivalis*) across the northern portion of their ranges. Conservation Genetics 15:1281–1298.

McBride, M.C., D.J. Hasselman, T.V. Willis, E.P. Palkovacs, and P. Bentzen. 2015. Influence of stocking history on the population genetic structure of anadromous Alewife (*Alosa pseudoharengus*) in Maine rivers. Conservation Genetics 1–15.

Messieh, S.N. 1977. Population structure and biology of Alewives (*Alosa pseudoharengus*) and Blueback Herring (*A. aestivalis*) in the Saint John River, New Brunswick. Environmental Biology of Fishes 2:195–210.

National Oceanic and Atmospheric Administration (NOAA). 2013. Endangered and threatened wildlife and plants; Endangered Species Act listing determination for Alewife and Blueback Herring. Federal Register 78:48944–48994.

Nielsen, E.E., L.A. Bach, and P. Kotlicki. 2006. hybridlab (version 1.0): A program for generating simulated hybrids from population samples. Molecular Ecology Notes 6:971–973.

Palkovacs, E.P., D.J. Hasselman, E.E. Argo, S.R. Gephard, K.E. Limburg, D.M. Post, T.F. Schultz, and T.V. Willis. 2014. Combining genetic and demographic information to prioritize conservation efforts for anadromous Alewife and Blueback Herring. Evolutionary Applications 7:212–226.

Peakall, R., and P.E. Smouse. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Quinn, T.P. 1993. Biological interactions of natural and enhanced stocks of salmon: A review of homing and straying of wild and hatchery-produced salmon. Fisheries Research 18:29–44.

Ramasamy, R.K., S. Ramasamy, B.B. Bindroo, and V.G. Naik. 2014. STRUCTURE PLOT: A program for drawing elegant STRUCTURE bar plots in user friendly interface. SpringerPlus 3. Accessed 15 April 2015.

Rosenberg, N.A. 2004. distruct: A program for the graphical display of population structure. Molecular Ecology Notes 4:137–138.

Rousset, F. 2008. genepop'007: A complete re-implementation of the genepop software for Windows and Linux. Molecular Ecology Resources 8:103–106.

Saunders, R., M.A. Hachey, and C.W. Fay. 2006. Maine's diadromous fish community. Fisheries 31:537–547.

Stabell, O.B. 1984. Homing and olfaction in salmonids: A critical review with special reference to the Atlantic Salmon. Biological Reviews 59:333–388.

Stewart, G., J.M. Caldwell, A.R. Cloutier, and L.E. Flight. 2006. Water resources data maine water year 2005. US Geological Survey, Augusta, ME. Available online at https://pubs.usgs.gov/wdr/2005/wdr-me-05-1/. Accessed 28 March 2015.

Taggart, J.B., R.A. Hynes, P.A. Prodöuhl, and A. Ferguson. 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. Journal of Fish Biology 40:963–965.

Van Oosterhout, C., W.F. Hutchinson, D.P.M. Wills, and P. Shipley. 2004. micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535–538.

Waters, J.M., J.M. Epifanio, T. Gunter, and B.L. Brown. 2000. Homing behaviour facilitates subtle genetic differentiation among river populations of *Alosa sapidissima*: Microsatellites and mtDNA. Journal of Fish Biology 56:622–636.

Weston, K.A., S.S. Taylor, and B.C. Robertson. 2016. Identifying populations for management: Fine-scale population structure in the New Zealand Alpine Rock Wren (*Xenicus gilviventris*). Conservation Genetics 17:691–701.

Wong, M.W., and D.W. Townsend. 1999. Phytoplankton and hydrography of the Kennebec estuary, Maine, USA. Marine Ecology Progress Series 178:133–144.