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GENETIC STRUCTURE AND mtDNA DIVERSITY OF *FUNDULUS HETEROCLITUS* POPULATIONS FROM POLYCYCLIC AROMATIC HYDROCARBON-CONTAMINATED SITES

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Abstract—Genetic structure and diversity of mummichog (*Fundulus heteroclitus*) populations were investigated using mitochondrial DNA (mtDNA) sequences. Forty-six haplotypes were identified among 208 mummichog from the Elizabeth and York Rivers in Virginia, USA. No evidence of decreased gene or nucleotide diversity for mummichog from polycyclic aromatic hydrocarbon (PAH)–contaminated sites was observed. However, based on mtDNA data from 17 sites, a significant correlation (Mantel analysis, $p = 0.035$) was noted between genetic distance (F_{ST}) and PAH concentration but not between genetic distance and geographic distance. Mummichog from the most heavily PAH-contaminated site, Atlantic Wood (AW), were genetically distinct from those of other Elizabeth River sites. At AW, high frequencies of several divergent haplotypes were observed that were more closely allied to the northern mummichog than to the more abundant southern form in the Chesapeake Bay. These data suggested that a locally stable population existed at the AW site. This conclusion is consistent with the observation that mummichog from the AW site display enhanced tolerance to PAH contamination relative to mummichog from noncontaminated sites. Conclusions about gene diversity and the correlation between genetic distance with site differences in PAH concentrations were also consistent with those from tandem genetic analyses based on allozymes.

Keywords—Population Genetics Fish Polycyclic aromatic hydrocarbons DNA

INTRODUCTION

Genetic change associated with environmental toxicants can result from several processes, including selection, drift, mutation, and gene flow. It follows that studies to determine toxicant effects to population genetic structure and diversity should provide information about as many of these processes as possible in order to fully understand the population-level consequences of contaminant exposure. Molecular genetic techniques offer an effective way for gathering such evidence.

The highly modified Elizabeth River in Virginia, USA, has elevated concentrations of polycyclic aromatic hydrocarbons (PAHs), tributyltin, and other toxicants in water, sediments, and organisms [1,2]. Contamination is extremely high at a Superfund site adjacent to the former Atlantic Wood Industries (AW) wood treatment plant. The AW site has sediment PAH concentrations more than 2,500 times higher than those of other sites along the river. Still, high numbers of mummichog (*Fundulus heteroclitus*) are consistently present at the AW site.

Mummichog inhabiting contaminated sites can exhibit enhanced tolerance (e.g., to mercury [3] or dioxin [4]), and Williams [5] suggested that this might be the case for AW mummichog. Polycyclic aromatic hydrocarbon concentrations in AW sediments were toxic or stressful to mummichog from noncontaminated localities [5–8]. Exposure of mummichog to high PAH concentrations was associated with a high prevalence of liver lesions and abnormal liver foci [6]. Williams [5] reported that AW mummichog tolerated levels of PAH that resulted in 100% mortality of mummichog from a reference locality on the nearby York River. These results suggested the presence of genetically based, enhanced tolerance in the AW population, although acclimation or maternal effects could not be eliminated as alternate explanations. Recently, the genetically based tolerance explanation was tested and accepted for AW mummichog by Ownby et al. [8]. Mummichog embryos from laboratory-reared AW mummichog had a much lower prevalence of cardiac terata than mummichog from other Elizabeth River and York River sites when exposed to sediment from the AW locality. Taken together, these observations provided strong evidence that AW mummichog have a genetically based, increased tolerance to PAH relative to mummichog from reference sites. The presence of this genetically based tolerance suggests that migration from sensitive populations was rare and/or that tolerance selection at the AW site was strong.

A recent study of allozyme variation indicated that mummichog from the AW site were genetically distinct from mummichog collected from other Elizabeth River sites [2]. This genetic distinction was maintained despite an estimated effective migration rate in this section (Southern Branch) of the Elizabeth River of 9.6 and 17.5 migrants/generation for juveniles and adults, respectively. (Ayala [9] suggests that random drift is unlikely to determine allele or haplotype frequencies under such high-migration conditions.) Also, in a regional landscape context, genetic distances between 17 Elizabeth and York River mummichog populations were correlated significantly with site differences in PAH concentrations but not with the geographic distances between sites. No evidence of reduced genetic diversity related to PAH contamination was observed despite this correlation, the genetic distinctness of the AW population, and the implied strong selection at the AW site.

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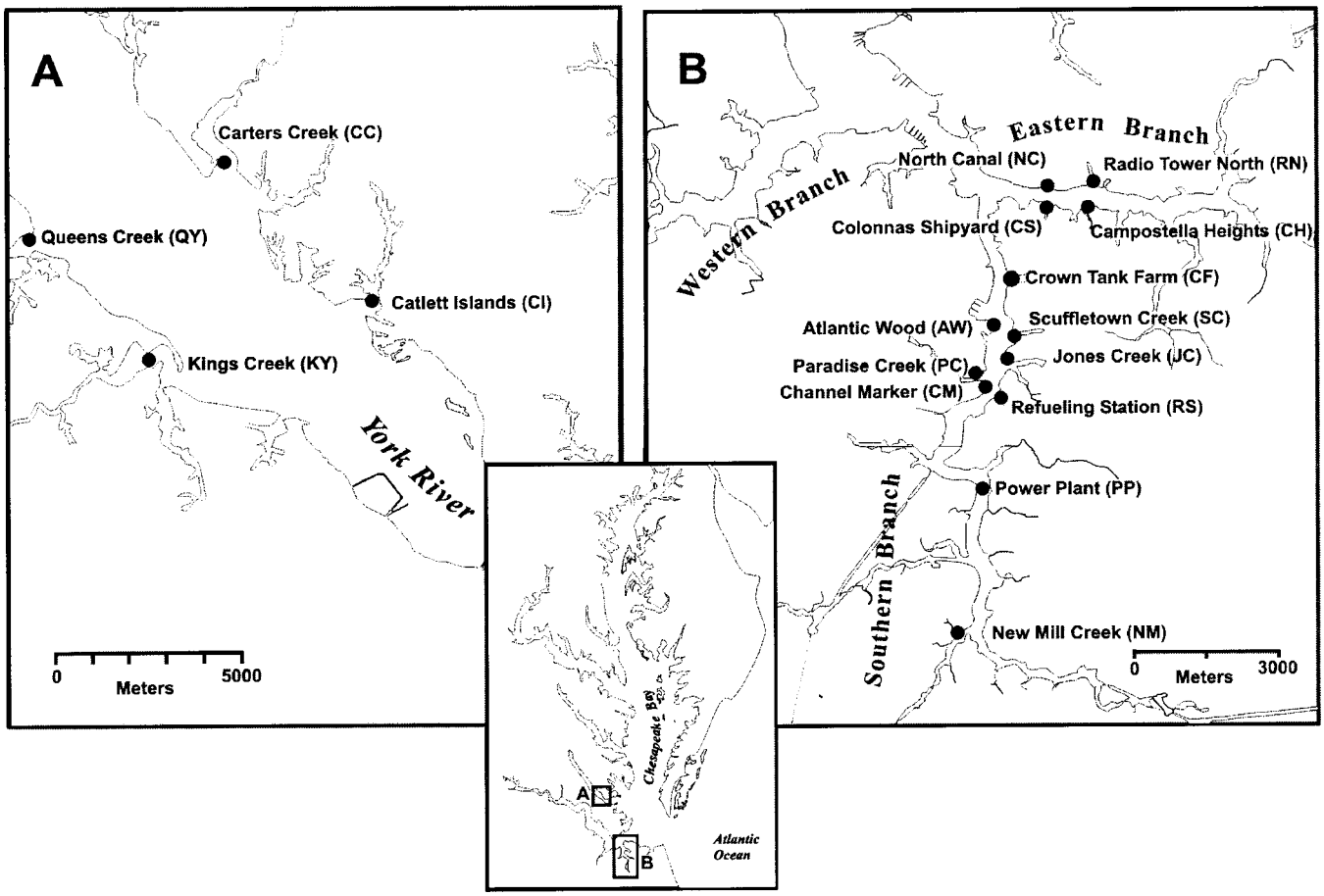

Fig. 1. Collection localities for mummichog along the Elizabeth and York Rivers (VA, USA). Distance over water between the Crown Tank Farm (CF) and New Mill Creek (NM) localities is approximately 12 km.

Here we report results from mtDNA sequencing for mummichog taken from the same lower Chesapeake Bay sites, including AW. These analyses were done to add or detract support for the conclusions from the tandem genetics analysis based on allozymes. The sequenced mtDNA segment includes portions of cytochrome B and the d-loop (S. Cohen, Harvard University, Boston, MA, USA, personal communication). The d-loop is the major noncoding region of the mtDNA and, because of reduced functional constraints, a region with fast rates of evolution.

Specifically, this study was done to determine whether genetic structure and diversity conclusions reached in the allozyme study would also be reached using a mtDNA marker. Comparable results with the mtDNA data would strengthen prior conclusions that mummichog in the heavily contaminated AW site represent a semi-isolated population, that mummichog genetic distance was correlated with differences in sediment PAH concentrations, and that genetic diversity did not decrease with increasing PAH contamination.

METHODS

Fish collection

Mummichog were collected from a total of 17 Virginia sites. Mummichog were collected initially in October 1998 along a 12-km reach of the Southern Branch of the Elizabeth River (Fig. 1). Each of the nine sites was a discrete habitat patch separated from the others by habitat unsuitable for mummichog. Eight more sites were sampled in October 2000 from the Eastern Branch of the Elizabeth River and from the nearby York River. The primary reason for the additional sampling was to increase the number and range of sites relative to geographic distance and PAH concentrations. Figure 2 shows that the 17 collection sites differed markedly in sediment PAH contamination. Also, because York River populations have been used in the past as reference populations in studies of

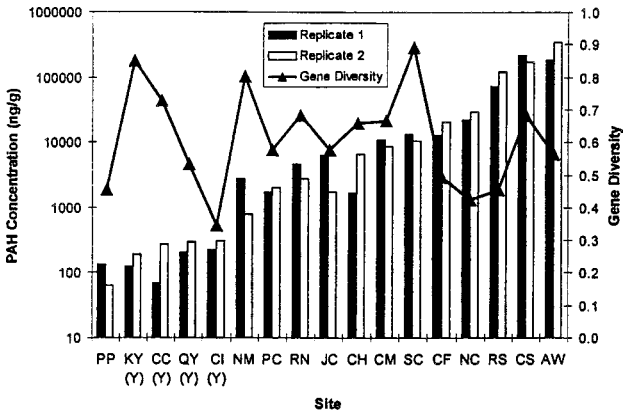

Fig. 2. Sediment polycyclic aromatic hydrocarbon (PAH) concentrations (ng/g dry wt of sediment) at each site. Results for duplicate samples are shown as vertical bars for each site. The letter Y below a site abbreviation indicates a York River site (VA, USA). Mummichog gene diversity (see also Table 2) is shown. See Figure 1 for site abbreviations.

Elizabeth River mummichog, a secondary intent of the expanded survey was to assess the appropriateness of using York River reference populations in studies of Elizabeth River mummichog. Approximately 60 of the largest adults (mean ± standard deviation: 7.3 ± 3.0 g wet wt) were collected from each site for genetic analysis. Ten to 15 of these were randomly selected per site for mtDNA sequencing.

Mummichog in the Chesapeake Bay region include both southern and northern forms of *F. heteroclitus* [10]. To facilitate identification of northern-form mummichog, whole genomic DNA preparations of northern mummichog from four New Bedford Harbor, Massachusetts, USA, sites (courtesy of Diane Nacci, U.S. Environmental Protection Agency, Atlantic Ecology Division, Narragansett, RI, USA) were obtained for comparison with Elizabeth River samples. One Massachusetts site was a polychlorinated biphenyl–contaminated Superfund site studied by Nacci et al. [11], and the others were nearby reference sites.

Sediment collection and analyses

Sediments were collected as described in Mulvey et al. [2]. Briefly, three grab samples were obtained from *F. heteroclitus* habitat at each site and the sediments homogenized to produce one composite sample. This process was repeated, and the resulting duplicate composite samples from each site were analyzed for PAH.

Sediments were analyzed for PAH using the protocol of Greaves et al. [12]. Briefly, sediments were freeze-dried, spiked with surrogate standards, and extracted with dichloromethane by accelerated solvent extraction. The resulting extracts were fractionated by gel permeation chromatography and silica gel and analyzed for aromatic or heterocyclic compounds by capillary gas chromatography with flame ionization detection and gas chromatography/mass spectrometry in the full-scan electron ionization mode. Blank samples, duplicates, and standard reference materials were analyzed simultaneously with environmental samples to ensure data quality. Aliquots of each sediment sample were also analyzed for total organic carbon to allow normalization of PAH concentrations to sediment carbon content. Carbon-normalized PAH concentrations were used in statistical analyses.

Total PAH concentrations were calculated as done in the appendix of Horness et al. [13]. Low-molecular-weight PAHs included biphenyl, naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, acenaphthalene, fluorine, phenanthrene, 1-methylphenanthrene, and anthracene. High-molecular-weight PAHs included fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene, benzo[*e*]pyrene, perylene, and dibenzo[*a,h*]anthracene.

DNA analyses

Total genomic DNA was extracted from muscle tissue with the phenol-chloroform method [14] followed by ethanol precipitation. Samples were resuspended in tris-ethylenediaminetetraacetic acid buffer or water. A segment of the mitochondrial genome was amplified using the polymerase chain reaction. For 50-μl reactions, the following were used: approximately 100 ng genomic DNA, 5 μl Promega® (Madison, WI, USA) buffer, 1 μl deoxynucleotide triphosphates (dNTP), 0.5 μl primer 1 and 0.5 μl primer 2, and 0.25 μl Taq (Promega Kit). The reaction profile was 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, 65°C for 3 min, and a 7-min final extension at 65°C. The two primers were 5′ CAT ATT AAA CCC GAA TGA TAT TT 3′ and 5′ ATA ATA GGG TAT CTA ATC CTA GTT T 3′. Following amplification, fragment size (~1,800 bp) was verified using 1% (w/v) agarose gel electrophoresis. Amplified products were cleaned with Microcon® filters (Millipore, Bedford, MA, USA). Cleaned products were sequenced using the 5′ CAT ATT AAA CCC GAA TGA TAT TT 3′ primer at the Molecular Genetic Instrumentation Facility at the University of Georgia (Athens, GA, USA).

The DNA sequences were edited and aligned using Genetool® (Ver 1) software (BioTools, Edmonton, AB, Canada). Sequence data were then analyzed using Arlequin Version 2.000 [15] to describe genetic diversity and divergence among samples. Arlequin [15] calculated nucleotide and gene diversity estimates and pairwise distances. Arlequin [15] was also used to assess geographic subdivision (analysis of molecular variance) and to test for significant correlations (Mantel analyses) between the matrices of genetic distance, site differences in sediment total PAH concentration (normalized to total organic carbon), and geographic distance. Relationships among haplotypes were determined using maximum parsimony (PAUP, Ver 4) [16].

RESULTS

PAH in river sediments

Analysis of the nonpolar aromatic fraction showed a predominance of unsubstituted and substituted PAH. Total PAH concentrations (calculated as done in the appendix of Horness et al. [13]) ranged from a low of approximately 99 ng/g dry weight at the power plant (PP) site to a high of 264,114 ng/g dry weight at the AW site (Table 1). Stations were arranged in Figure 2 from lowest to highest to illustrate the concentration gradient as well as the wide range in PAH contamination found among the sites. Duplicate sediment samples from each site showed that variability within a site was low relative to that among sites.

mtDNA variation

A 450-bp segment in the mtDNA was obtained for 208 Virginia and 11 New Bedford mummichog samples. Fifty haplotypes were observed (Fig. 3). Analysis of molecular variance for the lower Chesapeake Bay sites indicated that 95% of the total genetic variation was within-site variability. Only 1% of the variability was attributable to variation among the site groupings of Elizabeth River Southern Branch, Elizabeth River Eastern Branch, and York River. Four percent of the total genetic variability was variation among sites within each grouping. Associated fixation indices were $F_{SC} = 0.03928$, $F_{ST} = 0.04865$, and $F_{CT} = 0.00975$.

The number of observed haplotypes differed among Virginia sites from a high of 8 at the Scuffletown Creek (SC) and Kings Creek (KC) sites to a low of 3 at the AW and Catlett Islands (CI) sites (Table 2). The SC and KC sites received discarded mummichog from bait buckets of the many fishermen who frequent these public areas. This likely contributes to the high number of haplotypes observed at these sites. Among the New Bedford samples, only one haplotype was observed at two of the reference sites, and four haplotypes were observed at the polychlorinated biphenyl–contaminated site.

Relationships among haplotypes are illustrated as the network drawn in Figure 4. Haplotype 1, the common southern haplotype, was present at all Elizabeth and York River sites. The common northern haplotype (haplotype 30) was observed

Table 1. Sediment polycyclic aromatic hydrocarbon (PAH) concentrations and qualities at the sites on the Southern Branch of the Elizabeth River (ER-SB), Eastern Branch of the Elizabeth River (ER-EB), and the York River (York), Virginia, USA. Values for sediment qualities are provided for duplicate field samples for each site. Organic carbon content and PAH concentrations were used to estimate carbon-normalized PAH concentrations in statistical analyses

River	Site	Total organic carbon (%)		PAH (ng/g dry wt)	
ER-SB	Power Plant (PP)	0.16	0.14	63	135
	Paradise Creek (PC)	1.64	1.87	1,717	2,023
	Jones Creek (JC)	0.89	0.86	1,690	6,220
	New Mill Creek (NM)	0.90	2.44	777	2,806
	Channel Marker (CM)	0.21	0.30	8,527	11,019
	Scuffletown Creek (SC)	5.05	4.41	10,472	13,202
	Crown Tank Farm (CF)	5.08	6.62	12,934	20,660
	Refueling Station (RS)	3.94	20.40	70,855	121,374
	Atlantic Wood (AW)	3.80	9.86	182,528	345,700
ER-EB	Campostella Heights (CH)	2.23	2.34	1,630	6,615
	North Canal (NC)	5.94	6.23	22,021	29,242
	Radio Tower North (RN)	1.56	1.57	2,721	4,519
	Colonnas Shipyard (CS)	6.93	8.29	167,900	214,418
York	Catlett Islands (CI)	2.25	1.75	222	309
	Carters Creek (CC)	0.97	0.88	69	268
	Kings Creek (KY)	1.06	1.51	122	191
	Queens Creek (QY)	0.96	1.83	204	289

for 8 of the 11 New Bedford Harbor mummichog. Haplotype 1 comprised 64% (127/197) of the total observed haplotypes in Virginia mummichog. Forty-five additional haplotypes were observed in the Virginia mummichog (Fig. 3). Most of these haplotypes differed from the common haplotype 1 by a single base-pair change. Haplotypes 6, 15, and 17 were more than three steps divergent from the common haplotype 1 and shared several base changes with the northern haplotypes.

The AW site was distinct from most other sites in the proportions of the different haplotypes present (Fig. 4). Although the common southern haplotype 1 was present at AW, five of the 15 AW mummichog analyzed were haplotype 2, a haplotype two base-pair changes distant from haplotype 1. Another AW haplotype (6) was more akin to the northern form than to the common southern form. The New Mill (NM) site was also distinct because several NM haplotypes were similar to the northern form. These results for NM were consistent with the observations that the northern form tends to be captured in estuary headwaters during the present interglacial period [10] and that NM was the furthest upstream sampling site (Fig. 1).

Mantel analysis generates correlations for two or more matrices and tests for statistical significance of the correlations [17]. Such analyses were done for the lower Chesapeake Bay sites to compare genetic distance (F_{ST} for site pairs), geographic distance (over-water distance between sites), and site differences in PAH concentrations in sediments. The PAH concentrations were normalized to organic carbon content, and the square root of the carbon-normalized PAH concentrations were used in the Mantel analysis. A significant correlation between genetic and geographic distance would suggest that isolation-by-distance mechanisms were important at the landscape scale of this study. A significant correlation between genetic distance and PAH concentration would suggest, but not prove, that PAH contamination influences genetic distance between site populations. No significant correlation was found between genetic distance and geographic distance (correlation coefficient = -0.0935, $p = 0.731$). This lack of evidence for isolation by distance was consistent with the high intersite migration rates calculated by Mulvey et al. [2] and tandem allozyme-based Mantel analyses [2]. A significant correlation (correlation coefficient = 0.3746, $p = 0.035$) was calculated between genetic distance and site differences in PAH contamination. This was also consistent with results of the tandem allozyme studies [2].

Genetic diversity

Gene diversity among sites ranged from 0.345 to 0.891 for the lower Chesapeake Bay sites with no apparent relationship to the level of PAH contamination (Fig. 2), nor was an apparent trend seen in nucleotide diversity relative to PAH contamination (Table 2).

DISCUSSION

Information useful for understanding the influence of toxicants on population genetics is currently inadequate for accurate assessment of population-level effects or risk. However, such information is rapidly accumulating about effects to the common, widespread, and well-studied mummichog. This species provides a valuable model for studies ranging from local to landscape scales and, consequently, was chosen for these studies of PAH contamination in the Elizabeth River.

All results from the mtDNA analyses were consistent with those from the tandem allozyme analyses. Mulvey et al. [2] found that genetic distance was significantly correlated with site differences in PAH contamination but not geographic distance between sites. The most contaminated site (AW) had a mummichog population with genetically based enhanced tolerance and was also distinct relative to allozyme allele (*Idh-2* locus) and mtDNA haplotype frequencies. These results were consistent with those of Kirchoff et al. [18], who documented changes in esterase allozymes downstream of a pulp mill discharge. The distinctness noted for the AW population was maintained despite estimated high migration rates that would, in the absence of selection, homogenize the genetic qualities of the Elizabeth River mummichog [9]. Estimated migration rates were consistent with those calculated for mummichog by Brown and Chapman [19] and Smith et al. [10]. We speculate

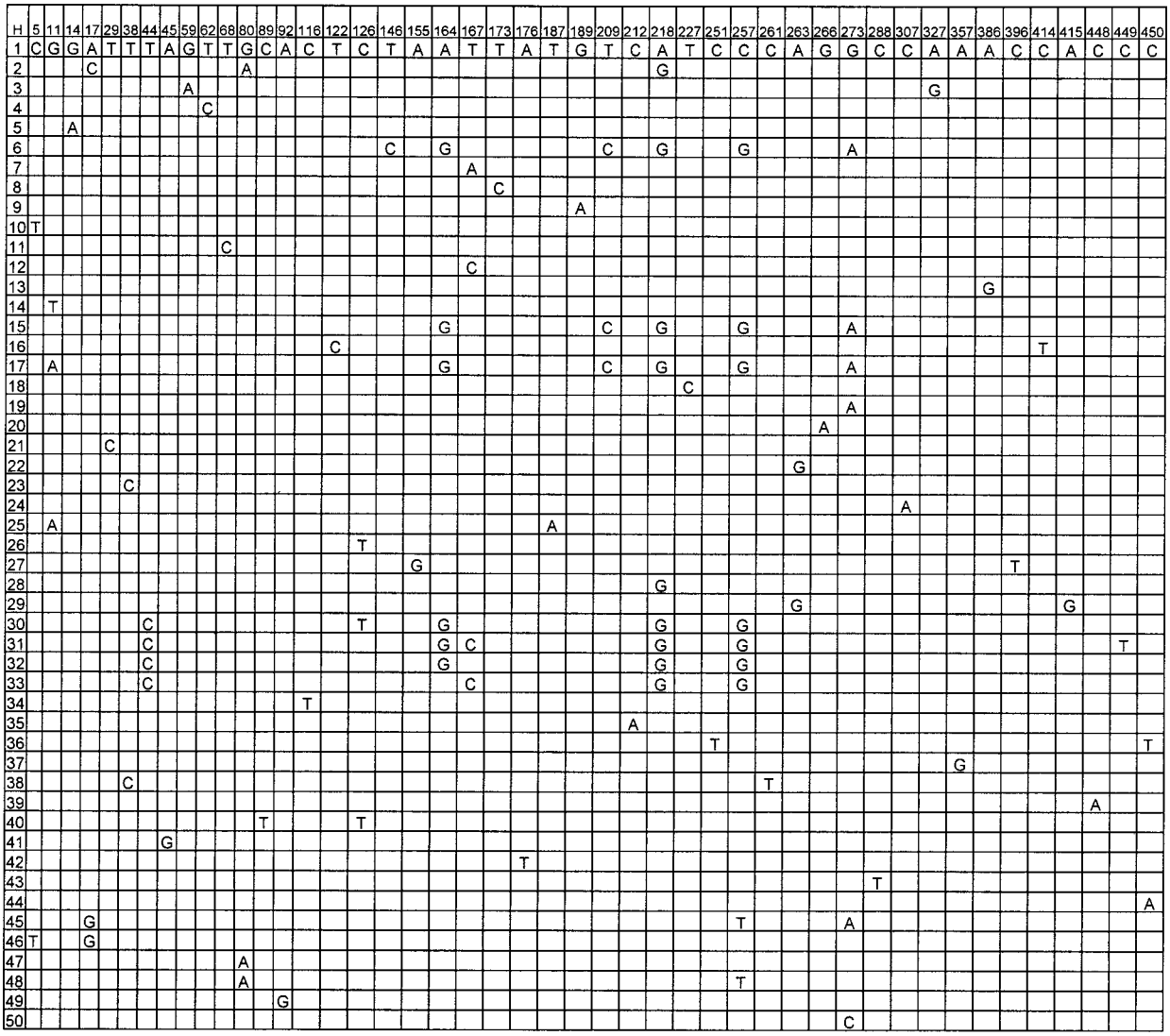

Fig. 3. The 50 haplotypes sampled from the Elizabeth River (VA, USA), the York River (VA, USA), and New Bedford Harbor (MA, USA). Base number in the DNA sequence is noted across the top of the figure, and the haplotype (H) is noted in the leftmost column. The New Bedford Harbor haplotypes are numbered 30 to 33. A = adenine, T = thymine, G = guanine, C = cytosine.

from these findings that strong selection, not drift, resulted in the distinct genetic qualities of AW mummichog. Based on the Mantel analyses for allozyme and mtDNA data, we also conclude that the genetic qualities of mummichog populations in a landscape context are influenced by the variation in PAH contamination within the habitat mosaic. Consistent with past work of Brown and Chapman [19] and Leamon [20], genetic drift due to isolation by distance was not significant at this geographic scale.

No trend was noted between organic carbon–normalized PAH concentrations and metrics of genetic diversity (Table 2 and Fig. 2). The high diversity at the SC and KC sites likely resulted from mummichog introduction from other sites by fishermen. There was a public park and boat ramp at the SC site, and because mummichog are a common baitfish, fishermen released mummichog from other areas into this site when emptying bait buckets at the end of the day. Similarly, the KC site was a site with easy access within a national park that experiences much shoreline fishing.

Introduction of stressors can reduce population size or eliminate sensitive individuals. On average, reduced effective population size can eventually reduce the genetic diversity of a population [21]. Similarly, the number of individuals carrying alleles into future generations is reduced by pollution-induced, genetic bottlenecks. Strong selection for one quality, such as toxicant tolerance, can also lower genetic diversity. Despite these potential mechanisms for reducing genetic diversity in exposed populations (see Newman [21] for more details), metrics of genetic diversity for allozyme and mtDNA data generated for PAH-exposed mummichog populations did not support the hypothesis that contamination compromises population evolutionary potential by lowering diversity. We speculate that, despite strong selection, the effective population size at the AW site remained sufficiently high to minimize any influence on genetic diversity, that high migration rates have adequately compensated for any loss of diversity, and/or that genetic diversity rebounded after sufficient adaptation to PAH contamination.

Other mechanisms could have been involved in maintaining genetic diversity. Genetic bottlenecks result in decreased genetic diversity on average [21]. By chance or by selection, individuals with high genetic diversity could be overrepre-

Table 2. Sample size (*n*), gene diversity, number of haplotypes, number of private haplotypes, and nucleotide diversity for a 450-bp segment of the mtDNA of mummichog from sites in the Southern Branch of the Elizabeth River (ER-SB), Eastern Branch of the Elizabeth River (ER-EB), and the York River (York), Virginia, USA

River	Site[a]	*n*	Gene diversity		No. of haplotypes	No. of private haplotypes	Nucleotide diversity	
			Mean	SE[b]			Mean	SE
ER-SB	PP	12	0.454	0.170	4	1	0.00148	0.00137
	PC	14	0.576	0.163	7	4	0.00222	0.00181
	JC	12	0.576	0.163	5	1	0.00148	0.00137
	NM	14	0.802	0.094	7	1	0.00493	0.00325
	CM	12	0.667	0.141	5	0	0.00313	0.00232
	SC	11	0.891	0.092	8	5	0.00323	0.00239
	CF	11	0.491	0.175	4	1	0.00396	0.00279
	RS	12	0.454	0.170	4	1	0.00111	0.00114
	AW	15	0.562	0.095	3	1	0.00474	0.00312
ER-EB	CH	14	0.659	0.123	5	1	0.00207	0.00170
	NC	13	0.423	0.164	4	1	0.00137	0.00129
	RN	12	0.682	0.148	6	3	0.00185	0.00159
	CS	12	0.682	0.148	6	2	0.00256	0.00120
York	CI	11	0.345	0.172	3	1	0.00080	0.00095
	CC	11	0.727	0.144	6	3	0.00234	0.00189
	KY	12	0.849	0.104	8	5	0.00296	0.00222
	QY	10	0.533	0.180	4	1	0.00133	0.00130

[a] See Table 1 for site abbreviations.
[b] SE = standard error.

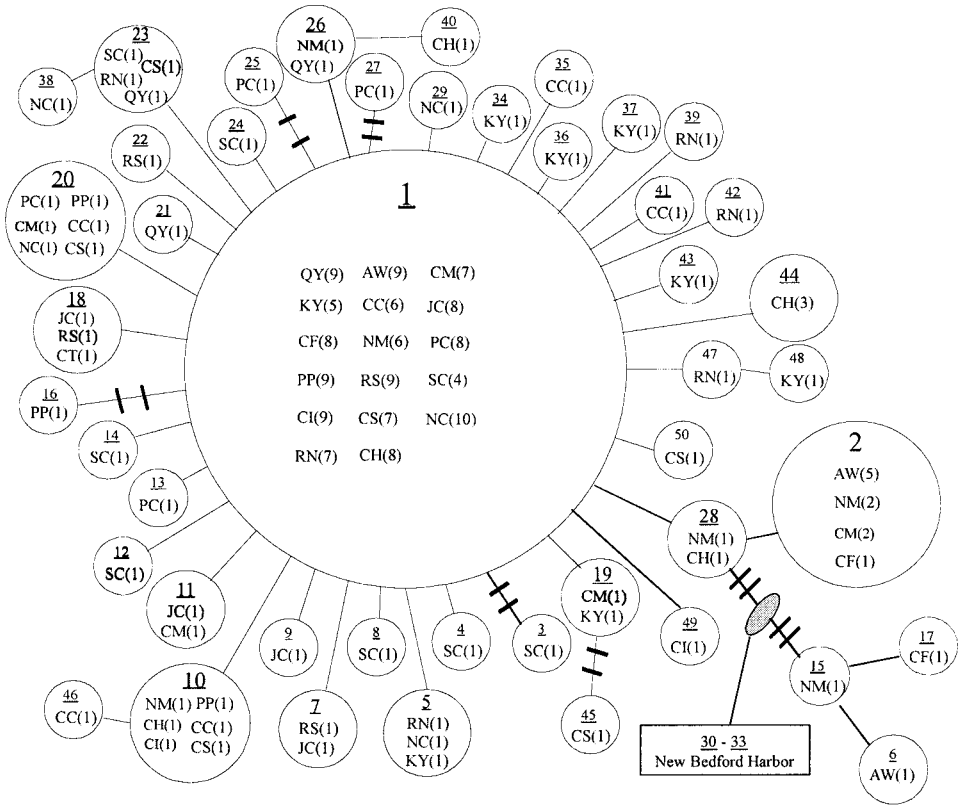

Fig. 4. The most parsimonious linkage of the 50 haplotypes including those from Virginia (circles) and New Bedford Harbor (MA, USA) (rectangle). Site identification code and number of individuals for each haplotype present at a site are noted below the haplotype number in each circle or rectangle. See Figure 1 for site abbreviations.

sented after the population passes through a bottleneck [21]. The population genetic diversity would increase in such a case. This mechanism is judged to be plausible, but less probable, than those described previously. Another explanation is increased mutation rates at the PAH-contaminated site. This alternate explanation was also judged to be plausible but less likely than the mechanisms proposed previously.

A secondary goal of this study was to assess the use of York River populations as reference populations in studies of Elizabeth River mummichog. The analysis of molecular variance results based on allozyme and mtDNA indicate very little genetic difference between York and Elizabeth River fish based on geographic distance. Genetic evidence suggested that the York River was an acceptable reference area with low PAH contamination.

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