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Evidence of Altered Gene Flow, Mutation Rate, and Genetic Diversity in Redbreast Sunfish from a Pulp-Mill-Contaminated River

CHRISTOPHER W. THEODORAKIS,*,† KAI-LIN LEE,‡ S. MARSHALL ADAMS,‡ AND C. BRANDON LAW§

Department of Biology and Environmental Sciences Program, Southern Illinois University, Edwardsville, P.O. Box 1651, Edwardsville, Illinois 62026, Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6036, and Department of Biology, Texas Tech University, Lubbock, Texas, 79409

To determine effects of pulp mill effluent on population genetic structure, redbreast sunfish (*Lepomis auritus*) were collected from several sites along the Pigeon River, NC, as well as from reference sites. Previous studies found effects on molecular, biochemical, physiological, population, and community level endpoints in these populations. The population genetic structure of these fish was determined using the randomly amplified polymorphic DNA (RAPD) technique. The level of genetic diversity was higher in the Pigeon River populations than in the reference populations. Genetic distances among populations could not be explained by drainage patterns and may have been altered by contaminant exposure. Phylogeographic analysis, maximum likelihood analysis, and assignment tests suggested that there were fewer emigrants and more immigrants in the contaminated sites than in the reference sites, suggesting that the contaminated sites may harbor "sinklike" populations. Finally, a "terminal branch amplitype" analysis (neighbor-joining and minimum-spanning trees) and maximum likelihood analysis indicated that there may be an elevated mutation rate in the polluted sites. Thus, the genetic diversity (within and among populations) in the Pigeon River populations may have been affected by altered gene flow and mutational processes as a result of pulp mill effluent discharge.

Introduction

The effect of environmental contaminants on population genetic structure has been the subject of many recent studies (*1*−*26*). Environmental contamination can influence population genetic structure via genetic bottlenecks, genetic adaptation to contaminants, alternations in gene flow, or enhanced mutation rate (see refs *27*−*30* for reviews). Such effects are significant because alterations in genetic diversity can affect the evolutionary plasticity, growth rates, sustainability, and probability of extinction of affected populations (*27*−*30*). In addition, alterations in population genetic structure may reflect perturbations of ecological parameters that are otherwise difficult to measure, i.e., population dynamics, recruitment, long-range/long-term dispersal, and source/sink or extinction/recolonization dynamics (*27*−*30*).

Recently, there has been increased interest in incorporating phylogeographic and landscape genetic analyses into assessments of pollution on genetic diversity (*4*−*7*). Phylogeography integrates the geographic or spatial distribution of genotypes (or alleles) and their phylogenetic (evolutionary) relationships (*31*). Landscape genetic analyses focus on spatially explicit genetic data without the need for delimiting discrete populations a priori and interpret patterns of genetic variation and gene flow in the context of landscape structure (*32*). Such analyses may aid in distinguishing between impacts of contaminants from biogeographic structuring or evolutionary history (*4*−*7*). Environmental contamination may also affect patterns of gene flow, immigration and emigration, and extinction/recolonization or source/sink dynamics. Hence, landscape genetic and phylogeographic analyses may provide insight into such effects (*4*−*7*).

To address these possibilities, the genetic structure of redbreast sunfish (*Lepomis auritus*) exposed to pulp mill effluents was examined using population genetic and phylogeographic approaches. The contaminants present in pulp and paper mill effluents include chlorinated phenols, dioxins, and other polyhalogenated aromatic hydrocarbons (PHAHs), resin acids, and polycyclic aromatic hydrocarbons (PAHs) (*33*). Chronic exposure to these chemicals may lead to genotoxic effects, endocrine disruption, and immune dysfunction (*34*−*36*). This can affect fitness components (growth, survival, reproduction, etc.) of resident populations and ultimately result in population or community level effects in ecosystems impacted by pulp mill effluent (*34*−*36*).

Experimental Section

Study Site. The Pigeon River is a tributary to the Tennessee River located in North Carolina and Tennessee (Figure 1). This river has been significantly degraded since 1908 by contaminant discharges from paper mill operations (*34*−*36*). Since 1988, extensive chemical and biological studies found that redbreast sunfish showed a reduction in overall health, fitness, and population parameters (*34*−*36*). In addition, there was evidence that populations at the impacted sites may have been ecological sinks, sustained largely by influx from nearby noncontaminated sites (S. M. Adams, personal observation). The flow of water in this river has been diverted in one section (see dashed line, Figure 1) so that the sampling site at Pigeon River kilometer (PRK) 42 is in the old river channel and no longer receives contaminated water from upstream. Thus, PRK 42 serves as a less contaminated site within the Pigeon River basin that only receives water from noncontaminated tributaries. The bioindicator studies (*24*−*26*) have indicated that the degree of impact increased in the order of Little River = Little Pigeon River ≥ PRK 103 (upstream of the mill) > PRK 42 > PRK 27 > PRK 89. Consequently, the biological data indicates (at least partial) recovery at site PRK 42 since diversion of the main river channel.

Fish Collection. Redbreast sunfish were collected in 1994 by boat electroshocking at sites indicated in Figure 1. Catch per unit effort and sex ratio data were taken from Adams et al. (*36*). (These data were collected from the same sites and at the same time as samples used for the RAPD analysis.) DNA was extracted from red blood cells as described previously (*27*).

* Corresponding author phone: (618)650-5235; fax: (618)650-3174; e-mail: ctheodo@siue.edu.
† Southern Illinois University
‡ Oak Ridge National Laboratory.
§ Texas Tech University.

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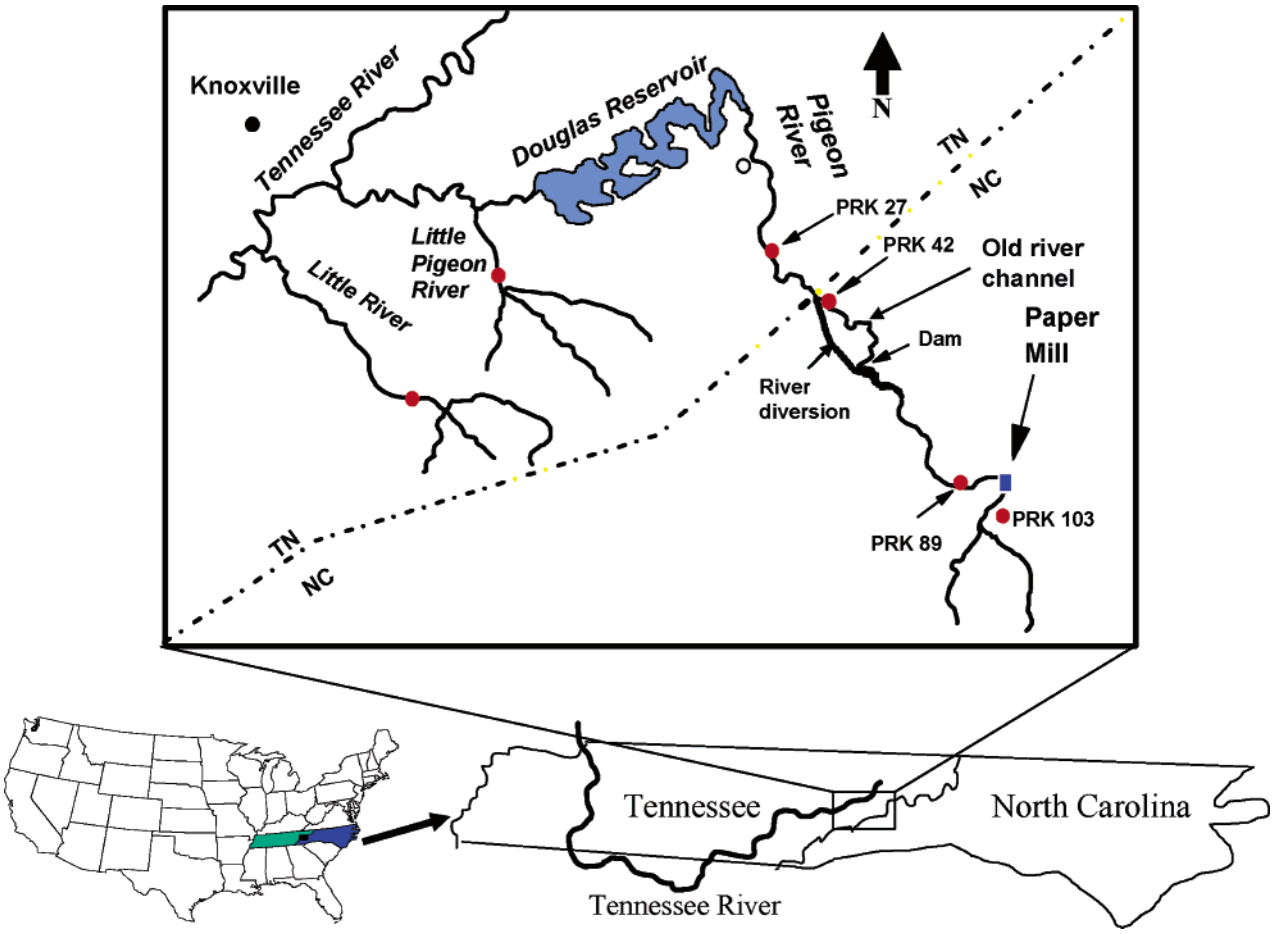

FIGURE 1. Sampling sites for redbreast sunfish populations collected from the Pigeon River and other rivers.

Genetic Analyses. The population genetic structure was determined using the randomly amplified polymorphic DNA (RAPD) as previously described (*37*, *38*), using 28 different primers. Quality assurance and control of banding patterns were as described previously (*37*). As in Lynch (*39*), data were "pruned" to remove bands with a frequency of less than 0.05.

Population genetic terminology is explained briefly below. For further explanation of terminology and the use of population genetic analysis in ecotoxicological studies, the reader is referred to Theodorakis and Wirgin (*28*) and to the "Definitions and Concepts" reported in the Supporting Information.

Fixation Indices. Fixation indices (Fst's) were used to determine if the sampling sites represented separate populations or if they were genetically indistinct locales within one population (*40*). If the Fst between two sampling sites was not statistically significantly different from 0, then the populations were regarded as being from the same genetic population. The Fst's were calculated using the program RAPD Fst (*41*) and the analysis of molecular variance (AMOVA) subroutine of the program Arlequin (*42*).

Dendrograms, Phylogeographic and Landscape Genetic Analysis. For this analysis, dendrograms were calculated among all populations and among individuals. A dendrogram is a branching diagram (sometimes referred to as a "tree") used to illustrate genetic relationships among populations, individuals, or genetic units (genotypes, alleles, haplotypes, or amplitypes). The dendrograms were used for several purposes. First, dendrograms of populations were used to determine if the genetic relationships followed those expected by isolation by distance—a model that assumes that the degree of genetic similarity decreases with increasing geographic distance (*40*). For this purpose, a neighbor-joining dendrogram among populations was calculated using the program RAPD Dist (*43*). A second method of determining genetic relationships among populations was to use the matrix of RAPD band frequencies for all populations to calculate a nonmetric multidimensional scaling analysis—a ordination technique that can graphically plot multivariate data on a two-dimensional plot.

Besides examining relative relationships among populations, dendrograms were used to determine relationships among all amplitypes. (An "amplitype" is a specific RAPD banding pattern (or "fingerprint") that is a reflection of the underlying genotype.) For this study, each fish had a unique amplitype. A neighbor-joining dendrogram among all individual amplitypes was calculated using the program RAPD Plot (*44*). Redbreast sunfish purchased commercially (Jonah's Aquarium, Thousand Oaks, CA) were used as the outgroup. A second analysis was conducted by constructing a minimum-spanning tree. This analysis uses specific algorithms to connect all genotypes (or in this case, amplitypes) by minimizing the sum of the lengths of all branches in the tree. The program Arlequin was used to generate a minimum-spanning tree among the amplitypes (*42*).

The amplitype dendrograms and trees were used for two additional purposes. First, the neighbor-joining trees were used to infer occurrence and direction (immigration vs emigration) of possible historical migration events among populations following Theodorakis et al. (*4*). These were used to calculate immigration/emigration ratios for each population. Differences in immigration/emigration ratios among populations were tested using a χ^2 test. Second, the number of tip amplitypes for each population was calculated from the neighbor-joining and minimum-spanning trees (*4*). The

assumption was that the tip amplitypes would be the most recently derived, and any new amplitypes arising due via mutation would be located at the tips of the trees (*45*). Differences in the percent of tip amplitypes among populations were tested using the χ^2 test.

Assignment Test. RAPD data were also used to run assignment tests according to Paetkau et al. (*46*). The percentage of misassigned individuals in each population were calculated, and permutation tests were used to test if the number of misassigned individuals for each pair of populations was statistically significantly different from zero (*47*). The percentage of misassigned individuals was then calculated for each population, and differences between populations in percent misassigned individuals were tested using Fisher's exact test. For each population, possible immigrants from populations not sampled were identified by individual log likelihoods (*47*). Differences in percent possible immigrants among populations were tested using the χ^2 test.

Maximum Likelihood Estimators. Two parameters that are commonly estimated using population genetic data are θ and γ. Theoretically, these two parameters are equivalent to $4N_e\mu$ and $4N_e$m, respectively, where μ and m are mutation and migration rates, respectively, and N_e is the effective population size. In population genetics literature, θ is used as an index of mutation rate or effective population size, while γ is used as an index of dispersal and gene flow.

Maximum likelihood estimates (MLEs) of θ and γ were calculated using the program MIGRATE (*48*), which allows for unequal population sizes and asymmetric migration rates. Because of the dominant nature of RAPDs, a minor modification of the data for input into the program was implemented. MIGRATE may still be useful for dominant markers that are scored as presence (1) or absence (0) of amplified bands, if the genotypes are coded as 0/0 for "band absent" amplitypes and 1/? (where "?" implies an unknown allele) for "band present" amplitypes (P. Beerli, Florida State University, personal communication).

In population genetic studies, the value θ can be used to estimate the mutation rate (μ) if the effective population size is known (*49*). However, both empirical and genetic methods for estimating N_e are difficult and/or require multiyear studies (*50*−*52*). Thus, catch per unit effort was used to calculate a qualitative, relative correlate of effective population size. A number of factors can influence effective population size (*50*), but the only one of these that was readily available from the current data was sex ratio. The effective population size is affected by sex ratio according to the formula $N_e = 4N_{ef}N_{em}/(N_{ef} + N_{em})$, where N_{ef} and N_{em} are the effective population sizes of females and males, respectively. Thus, catch per unit effort (CUE) was adjusted for the sex ratio according to the formula $\text{ACUE} = 4\text{CUE}_f\text{CUE}_m/(\text{CUE}_f + \text{CUE}_m)$, where CUE_f and CUE_m are the CUEs of females and males, respectively, and ACUE is the adjusted catch per unit effort. Therefore, ACUE was taken to be a qualitative, relative correlate of N_e.

Hence, θ was divided by ACUE (hereafter, θ/ACUE is referred to as the "mutation rate index"). Note that this is not an unbiased estimate of the true mutation rate but that this is rather a relative measure of mutation rate. It is only used here for comparative purposes, and it is assumed that is biased to the same extent and in the same direction for all populations sampled here.

To obtain a better indication of the relative patterns of immigration and emigration among sites, the γ ($4N_em$) values for each *ij* pair of populations (migration from population *i* to population *j*) was divided by the ACUE for population *j*. Again, this is not a quantitative estimate of m_{ij}, but it is assumed that the relative trends in m_{ij} among populations is better reflected by $\gamma_{ij}/\text{ACUE}_j$ than by γ alone. Total "immigration" was assessed by summing $\gamma_{ij}/\text{ACUE}_j$ across all *i*'s for each *j* population, and total "emigration" was assessed by summing across all *j*'s for each population.

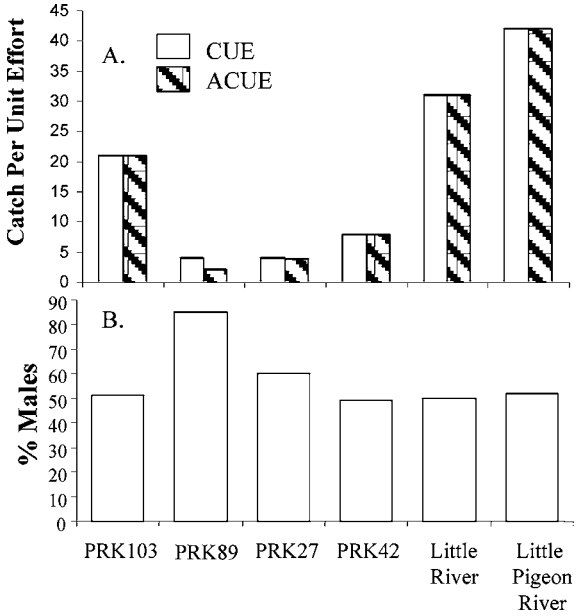

FIGURE 2. (A) Catch per unit effort (CUE) and adjusted catch per unit effort (ACUE) for redbreast sunfish populations collected from contaminated and reference sites in the Pigeon River and other rivers. (B) Sex ratio for the same populations.

Genetic Diversity. Genetic diversity was determined by using the dissimilarity index (*D*), $D = 1 - S$, where *S* is the average similarity index calculated between all pairs of individuals within the populations (*38*). The confidence intervals for *D* were calculated from the variance, and variance was estimated as described in Lynch (*39*).

Results and Discussion

Population Parameters. Catch per unit effort and sex ratios for each sampling site are indicated in Figure 2. The age structures of the populations are indicated in Figure 3 Data used to make Figures 2 and 3 were taken from Adams et al. (*26*), but these data were collected from the same sites and at the same time as the samples for the RAPD analysis presented here. At sites PRK 89 and PRK 27, the age structure was more heavily skewed toward larger, older individuals, while in the Little and Little Pigeon Rivers, it was more heavily skewed toward smaller individuals and young-of-the-year. For all population parameters, PRK 103 and PRK 42 were intermediate between the reference (Little and Little Pigeon Rivers) and contaminated (PRK 89 and PRK 27) populations.

RAPD Analysis: General. The RAPD primer sequences, number of polymorphic bands amplified by each primer, and molecular lengths of each polymorphic band amplified are also in the Supporting Information, Table S1.

The sample sizes for each of the sampling sites are as follows: PRK 103, 55; PRK 89, 55; PRK 42, 73; PRK 27, 46; Little Pigeon River, 43; Little River, 64. Several studies have demonstrated the limitations of RAPDs in analyses such as genetic diversity, neighbor-joining trees, and assignment tests (*53*−*55*). These authors have indicated that such limitations can be circumvented or minimized with large sample sizes and/or a large number of loci. Because the total number of loci used in the present study was 77 and the total number of individuals in the sample was 384, it was assumed that the biases due to limitations discussed in the papers cited above (*53*−*55*) are minimal.

The Fst statistics among populations using Nei's and AMOVA analysis both indicated that the only Fst that was not statistically different from 0 was between PRK 89 and 103

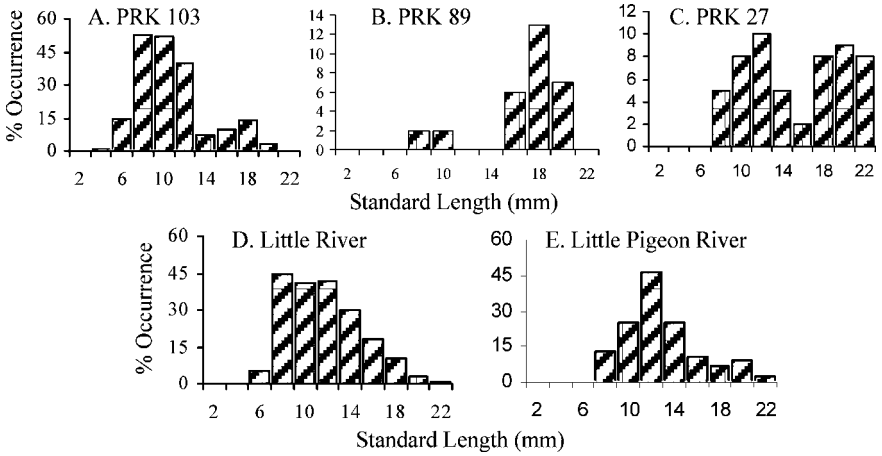

FIGURE 3. Standard length (snout to tip of caudal peduncle) and percent occurrence of each size class in redbreast sunfish collected from three sites in the Pigeon River, TN, and two nearby rivers.

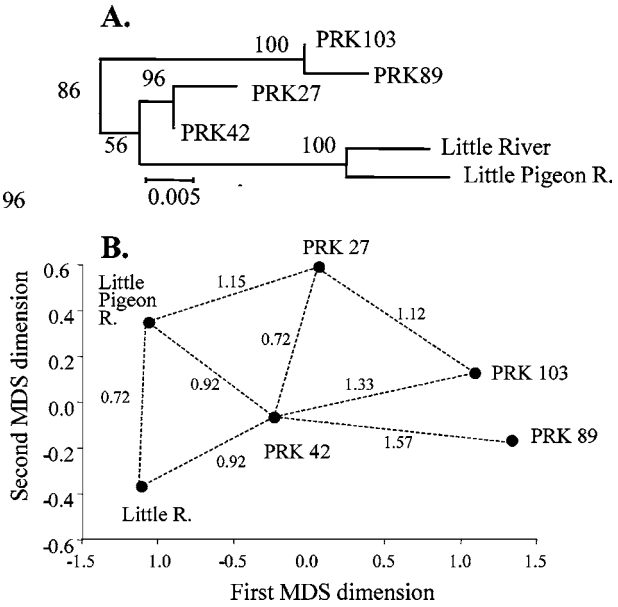

FIGURE 4. (A) Neighbor-joining dendrogram for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers. Numbers at the branch points represent bootstrap support for the branch points (based upon 1000 bootstrap replicates. The dendrogram is based upon the RAPD similarity index. (B) Nonmetric multidimensional scaling (MDS) results using band presence/band absence data for RAPD bands from the same populations. Group centroids (the centers of the distributions of data points) are plotted on the first and second MDS axes. Dashed double-headed arrows indicate Euclidean distances between centroids, calculated from the MDS coordinates.

(for the Nei's and AMOVA analyses, the Fst's were 0.007 and 0.001, respectively). This suggested that there is enough gene flow between these two populations that they are not genetically distinct. Therefore, these sites are considered to be a continuous population for the remainder of the analysis. In general, the other Fst's ranged from 0.030 to 0.207. For the sake of brevity, the other Fst's are reported in the Supporting Information, Table S2.

Genetic Relationships among Populations. Dendrograms of the populations (Figure 4A) indicated that (1) the PRK 42 and 27 populations were more similar to the non-Pigeon River sites than they were to PRK 89−103, contrary to what would be expected based on geographical distributions, (2) the PRK 89 site (downstream of the paper mill) seemed to be more derived than the PRK 103 (upstream) site, and (3) PRK 27 (more contaminated) was more derived than PRK 42 (less contaminated).

The results from the multidimensional scaling analysis are similar (Figure 4B). They indicate that the PRK 89 and PRK 103 sites are more divergent from the reference populations than are the other pigeon river sites and that site PRK 89 is the most divergent from the reference sites. It also shows that the PRK 27 site is more divergent from the reference sites than is PRK 42 (the old river channel site that may be undergoing recovery).

The genetic differentiation of the PRK 89−103 population from the other Pigeon River populations is probably influenced by the construction of dams, but this does not explain why the differences between PRK 89−103 and the other Pigeon River sites were greater than that between PRK 27/42 and the Little/Little Pigeon River. This is consistent with the hypothesis that these genetic differences were exacerbated by impacts of the paper mill. Other studies have also found enhanced genetic differentiation between contaminated and reference populations, possibly due to restricted gene flow as a result of the contamination (*56*).

Amplitype Dendrogram and Minimum-Spanning Tree. The dendrogram among all amplitypes is presented in Figure 5. Examination of the amplitype dendrogram indicates that the PRK 89−103 population tends to form a distinct clade, which is not apparent for the other populations (Figure 5). This is probably influenced by a barrier to gene flow (a dam) between the PRK 89−103 populations and the other populations. However, this is also consistent with the hypothesis that these patterns were also mitigated by contaminant effects (mutation, selection). For example, the PRK 89−103 population(s) are the closest to the paper mill, and there seems to more difference between the PRK 89−103 population and the other Pigeon River sites than those between the PRK 27/42 and the Little/Little Pigeon populations. This is contrary to the landscape patterns, which indicated that Pigeon River and the Little Pigeon/Little River are separated by one or more dams and considerable geographic distances (Figure 1).

Visual inspection of the minimum-spanning tree (Figure 6) reveals that sites PRK 42 and the Little River are fairly well segregated and clustered toward the center of the tree. The tips at the outer periphery are dominated by individuals from PRK 89−103, PRK 27, and the Little Pigeon River. Such a pattern could either be due to a recent range expansion and growth of these populations or de novo appearance of amplitypes. For the Little Pigeon River, the data presented below are more consistent with the former hypothesis, while for PRK 89−103 and PRK 27, the data presented below are more consistent with the latter. The topology of the tree also

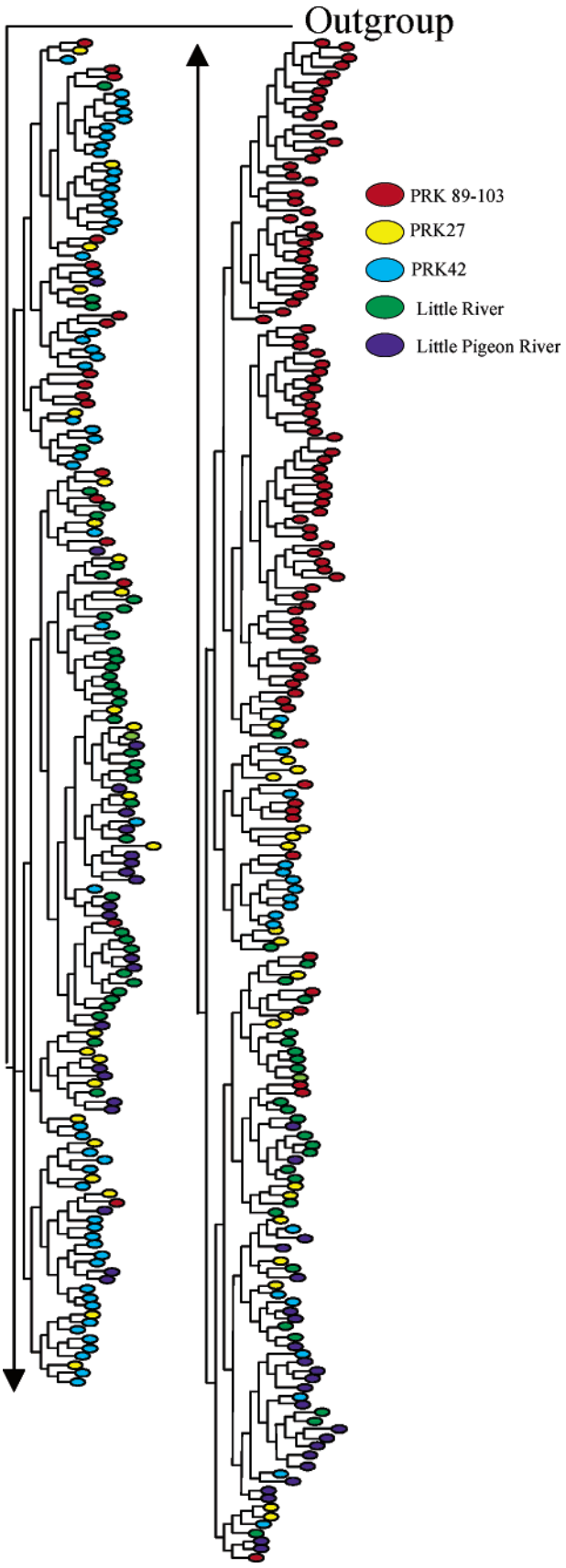

FIGURE 5. Neighbor-joining dendrogram for RAPD amplitypes, based upon a matrix of dissimilarity between each pair of amplitypes. Arrows indicate tip amplitypes. The outgroup (EPFC) was based on the RAPD banding pattern of a redbreast sunfish collected from a stream (East Fork Poplar Creek) that does not flow into the Clinch River.

argues against genetic drift as the sole factor in the divergence of PRK 89−103 from the other populations. The PRK 89−103 haplotypes are primarily derived (peripheral), and there is no reason to expect that drift would result in the preferential loss of ancestral (interior) haplotypes or preferential retention of derived haplotypes.

Indicators of Mutation Rate. There were three sets of data that were used to infer relative mutation rates for the redbreast sunfish populations: number of private alleles, the relative mutation rate inferred using the mutation index, and the number of tip amplitypes.

The band frequency data were inspected for the presence of private alleles, alleles which occur in one population or river that do not occur in any others. Private alleles could only be inferred from "band present" amplitypes because of the dominant nature of RAPDs. It was determined that there were 17 private ("band present") alleles, as indicated by bands that were found in one population or river and not in another (Table 1). In general, the number of private alleles increases in the order of Little River ≤ Little Pigeon River < PRK 42 < PRK 27 < PRK 89−103.

There are three possible explanations for the increased level of private alleles in the contaminated sites. (1) These patterns could be due to genetic drift. However, this would require loss of the "band present" alleles in all noncontaminated sites. (2) These bands may actually occur in the noncontaminated sites at a very low frequency, but they were simply not included in the samples from these populations by chance. However, in this case the frequency of these bands is still concordant with the degree of contaminant impact. (3) These bands could have arisen due to contaminant-influenced effects, such as de novo mutation or selection for rare genotypes. Other studies have found evidence of selection-driven differences among populations exposed to xenobiotics, with RAPD or other markers (*8*−*12*, *37*, *38*). An increased mutation rate is consistent with the increased number of private alleles with increasing levels of contamination. Previous studies have also found RAPD bands that were detected only in contaminated populations and not in the reference sites (*37*, *38*, *56*).

An additional line of evidence for an increased mutation rate is that the "mutation index" (i.e., θ/ACUE) was higher for PRK 89−103 and PRK 27 than those for the other sites (Figure 7A). These results are consistent with the hypothesis that the mutation rate is elevated in the Pigeon River (downstream of the papermill) and that the mutation rate decreases in the order of PRK 89−103 > PRK 27 > PRK 42 > Little Pigeon = Little River (Figure 5A). Recall that PRK 42 is in the old river channel, which is fed by uncontaminated streams, and thus would be less impacted than PRK 27.

The other line of evidence for and increased mutation rate in contaminated sites is that there were an increased number of tip amplitypes inferred from the amplitype dendrogram and the minimum-spanning tree in the PRK 89−103 and PRK 27 sites compared to the reference sites (Figure 7B).

Besides the contaminated sites, the Little Pigeon River also has an elevated percentage of tip amplitypes for the MST analysis (Figure 7B). It has been argued that the shape of a dendrogram may be influenced by whether a population is expanding or declining (*57*). In particular, excesses of tip genotypes may be expected in expanding population, because these populations are less likely to lose new mutant genotypes (*58*). The CUE data indicate that the Little Pigeon River is the largest sampled population, and so this does not rule out a recent expansion, but the same is not true for the contaminated sites, especially PRK 89 (Figure 2). In fact, if the number of tip amplitypes is normalized for CUE, then the data show a pattern similar to the bioindicators of adverse effects (*36*, *37*) (Figure 7C). If the number of tip amplitypes were solely

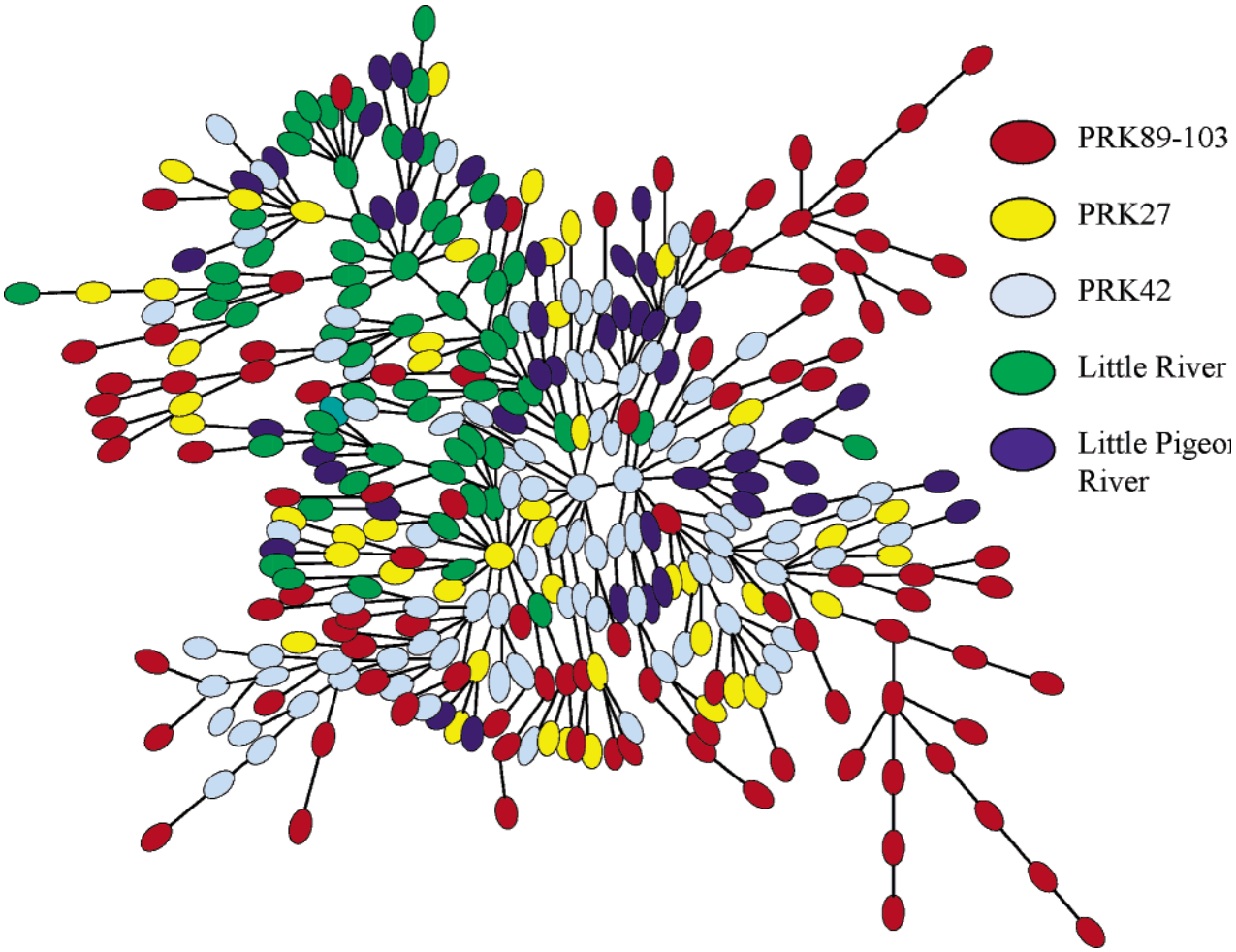

FIGURE 6. Minimum-spanning tree for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers.

due to differences in population size, then the tip amplitypes/CUE should be similar in all populations. In addition, size distribution data indicated that fewer individuals of smaller size classes were present at the contaminated sites (Figure 3). The relative numbers of individuals in the smaller size classes are often an indication of the rate of population growth, which also argues against a recent expansion in the contaminated sites. In fact, the demographic data suggest that there has been a recent decline in population size in the contaminated sites, so one would actually expect fewer tip amplitypes in the contaminated populations. Therefore, these data suggest that an increased number of tip amplitypes in the contaminated populations is not due to recent population growth.

There are two alternative hypotheses for an increased mutation rate in the contaminated sites. The first is that there are mutagenic contaminants in the paper mill effluent. This hypothesis is supported by a previous study that used the Ames assay to determine the mutagenicity of organic solvent extracts of redbreast sunfish tissues (*59*). It was found that the level of mutagenic substances in the tissues was greatest in sites immediately below the paper mill, and there was a mutagenic dose−response relationship (*59*). This indicates the presence of genotoxic and potentially mutagenic substances in these sites. The second alternative hypothesis is that there was a decrease in population size induced by paper mill effluent toxicity and that random mutations are more likely to accumulate in smaller, more inbred, populations (*60*). In addition, there is growing evidence that RAPD fingerprint patterns may be affected by somatic effects such as DNA damage, genomic instability, mutations, and chromosomal rearrangements (*61*−*63*). This could contribute to the apparent increase in mutation rates as well as increased occurrence of private alleles seen in the contaminated sites.

Gene Flow, Immigration/Emigration Ratios, and Source/Sink Dynamics. The amplitype dendrogram suggested that there were contaminant-influenced levels of gene flow, and this was supported by the assignment test and maximum likelihood analyses. For example, the immigration/emigration ratio, as calculated from possible dispersal events inferred from the dendrogram topography (*4*) was higher in the noncontaminated sites than that in the contaminated sites (Figure 8A), suggesting that there may have been processes similar to "source/sink" dynamics, and the contaminated sites were more similar to an ecological "sink", and the reference sites were more similar to an ecological "source". However, caution should be applied when using the topology of dendrograms to infer patterns of migration based upon coalescence theory or for that matter evidence for mutations based upon dendrogram topology, when using markers such as RAPDs. This is because the topology of nuclear markers could be affected by recombination (*64*) or the number of loci examined (*55*). Thus, any information gathered from such topologies should not be used by itself but must be supported by additional analyses as part of a larger "weight of evidence" assessment.

To this end, analyses from the assignment tests have also provided evidence of asymmetric gene flow between contaminated and noncontaminated populations. For example, the assignment test calculates the likelihood that the parents

TABLE 1. Frequency of "Private Bands"[a] for Redbreast Sunfish Populations Collected from Contaminated (PRK 89, PRK 27) and Reference Sites in the Pigeon River and Other Rivers

	population				
RAPD band[b]	PRK 89−103	PRK 27	PRK 42	Little Pigeon River	Little River
OPA-01_{1240}	0.09	0	0	0	0
OPA-04_{600}	0.16	0.04	0	0	0
OPA-07_{1140}	0.36	0.06	0	0	0
OPA-07_{880}	0.36	0.06	0	0	0
OPA-09_{890}	0.29	0.26	0.25	0	0
OPA-11_{2200}	0.20	0.06	0.04	0.11	0
OPA-11_{1850}	0.20	0.04	0	0	0
OPA-20_{870}	0.64	0.11	0.25	0	0
OPD-05_{2100}	0.24	0	0	0	0
OPD-05_{1200}	0	0.09	0.06	0.02	0.26
OPD-08_{820}	0.20	0.11	0.18	0	0
UBC-06_{920}	0.21	0	0	0	0
UBC-12_{1100}	0.22	0.04	0	0	0
UBC-14_{840}	0.29	0.09	0.10	0	0
UBC-30_{1500}	0.16	0	0	0	0
UBC-31_{1090}	0.18	0.17	0.25	0	0

[a] RAPD bands that are found in one population/sampling site and not in others. [b] RAPD band names are derived from the name of the primer used to amplify that band (e.g., OPA-01) followed by the molecular length of the band, in subscript. Primers beginning with "OP" were purchased from Operon Technologies (Alameda, CA) and were obtained from either RAPD primer kit A or kit D. Primers beginning with "UBC" were purchased from the University of British Columbia, Nucleic Acid and Protein Services Unit.

(or other ancestors) of an individual came from the population in which it was sampled versus another population from a different location (*47*). With this approach, it was found that the percent of misassigned individuals was greater in the contaminated populations than that in the reference populations (Figure 8B). If one assumes that at least some individuals that were misassigned had recent ancestors from a population that was different from the one from which they were sampled, then this would indicate that the contaminated populations were composed of more immigrant lineages than the reference populations. Table S4 also indicates that the number of individuals sampled from a contaminated site but assigned to a reference site (i.e., possible immigrant from that reference site) was, in general, greater than the reverse. This is especially true for populations PRK 27 (more impacted) and PRK 42 (less impacted), the two closest populations without a dispersal barrier between them, and hence the two populations for which recent dispersal would be likely to occur. The number of possible recent immigrants, both from populations that were sampled and populations that were not, were calculated using the assignment test, and it was found that the percent possible recent immigrants was greater for the contaminated sites, especially PRK 27, than that for the reference sites. (Note that the data for the number of individuals sampled from one population and assigned to another can be found in the Supporting Informations, Table S5).

Recall from the Experimental Section that the asymmetric values of γ ($4N_em$) were divided by the ACUE, to calculate a "migration index", and the migration index for immigration was divided by the migration index for emigration to calculate the immigration/emigration ratio. (The individual values of γ for each pair of populations can be found in the Supporting Information, Table S5.) With this approach, the immigration/emigration ratio was higher in the contaminated sites than that in the reference sites, with PRK 42 intermediate (Figure 8C). This suggests that the contaminated Pigeon River populations are more "sinklike" than the other populations, and the magnitude of this effect is influenced, at least in part, on the level of contaminant impact.

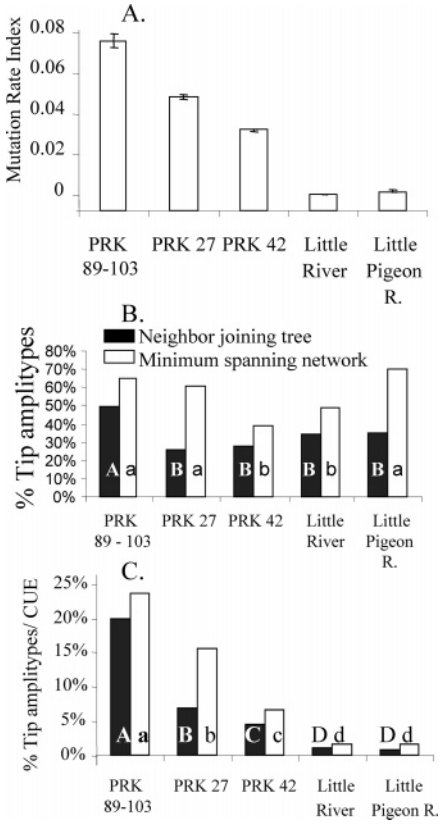

FIGURE 7. (A) Mutation rate index (θ/ACUE) for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers. $\theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per generation. ACUE $= 4CUE_fCUE_m/(CUE_f + CUE_m)$, where CUE_f and CUE_m are the catch per unit effort (CUE) of females and males, respectively. Error bars represent 95% confidence intervals. The value of θ is taken to be statistically significantly different between any two sites ($p < 0.05$) if the error bars do not overlap. (B) Percent individuals with tip amplitypes for redbreast sunfish from the same populations. Bars labeled with different letters are significantly different ($p < 0.05$, χ^2 test). The tip amplitypes were determined both for a neighbor-joining tree (based upon a matrix of RAPD similarity indices) and on a minimum-spanning tree (based upon RAPD band presence/absence data). (C) Data in part A normalized by catch per unit effort (CUE).

From a weight of evidence perspective, the overall pattern of immigration/emigration ratios is that the Pigeon River site PRK 27 has the most skewed immigration/emigration ratio, followed by PRK 89−103, PRK 42, and the other two river systems, respectively. It is not unexpected that the immigration/emigration ratio is higher for PRK 27 than that for PRK 89−103, because PRK 89−103 is nearer the headwaters of the Pigeon River, so there are more opportunities for dispersal from other sites into PRK 27 (both now and in the past). If true, then this would indicate that physical location and accessibility for potential immigrants would be a confounding factor in interpretation of immigration/emigration ratios.

Genetic Diversity. Relative levels of genetic diversity are reported in Figure 9. Partitioning of genetic diversity into diversity within and among populations, as revealed by AMOVA analysis, is reported in the Supporting Information (Table SM3).

Analysis of genetic diversity reveals two general trends. The first general trend is that there is a increase in genetic diversity in the contaminated sites relative to that of the reference sites (Figure 9). The increased genetic diversity is probably not due to increased population size or growth,

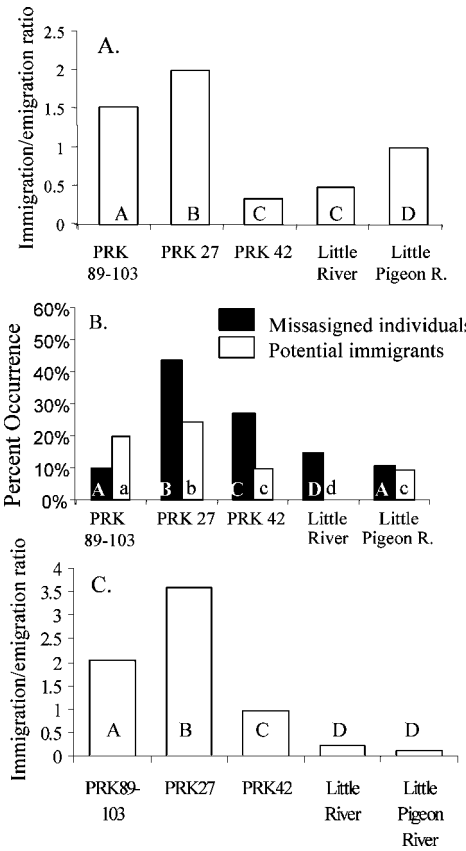

FIGURE 8. (A) Emigration/immigration ratios for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers. The direction of probable historic migration events (immigration vs emigration) was inferred from the amplitype neighbor-joining dendrogram. (B) Percentage misassigned (cross-assigned) individuals for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers, and percentage of individuals representing possible recent immigrants, as determined by the assignment test. (C) Immigration/emigration ratios calculated on the basis of a maximum likelihood estimator of asymmetric $4N_em$, where m, the migration rate from population B to population A, is calculated separately from migration from A to B. Data are presented for $4N_em$ and for $4N_em$/ACUE. ACUE = $4CUE_fCUE_m/(CUE_f + CUE_m)$, where CUE_f and CUE_m is the catch per unit effort (CUE) of females and males, respectively. In all cases, bars labeled with different letters were not significantly different ($p < 0.05$, χ^2 test).

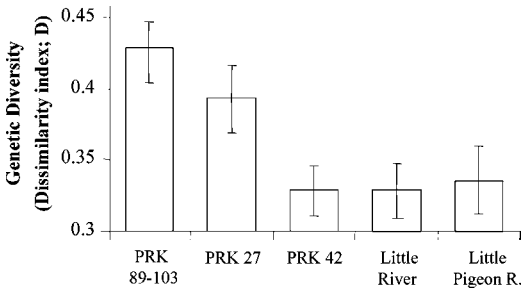

FIGURE 9. Genetic diversity for redbreast sunfish populations collected from contaminated (PRK 89, PRK 27) and reference sites in the Pigeon River and other rivers. Bars and error bars represent the average RAPD dissimilarity index with 95% confidence intervals, respectively.

because the CUE and size distribution data (Figures 2 and 7) indicate that these populations are not larger or more rapidly growing than the others. In addition, Adams et al. (*35*) argued that there was evidence of reduced recruitment into the contaminated Pigeon River sites. It could also be that the contaminated Pigeon River sites have more genetic diversity than the other sites due to past population bottlenecks in the reference populations, but there is no reason to suspect bottlenecks in the Little Pigeon and Little Rivers. Finally, population genetic diversity can be a function of population size, but the catch per unit effort data and the ACUE (assumed to be reflections of population density and effective population size, respectively; Figure 2) are not concordant with this explanation.

The fact that there was a greater amount of genetic variation in the contaminated than in the noncontaminated sites is contrary to what has been found in other studies with RAPDs (*13*−*16*). However, an increased genetic diversity has been found with RAPD markers in other species exposed to environmental contaminants (*17*, *38*). Increased genetic diversity in contaminated populations has also been found using other markers as well (*18*−*26*). For the present study, if the increased genetic diversity in contaminated sites is indeed due to contaminant effects, then there can be two (not mutually exclusive) explanations. The first is that asymmetric gene flow, with greater gene flow into contaminated populations than reference populations, may lead to greater genetic diversity because of immigrants with novel alleles. The second may have to do with creation of novel genotypes in the contaminated habitats. This may include processes related to genomic instability (point mutations, chromosomal rearrangements, gene amplifications). The RAPD technique has been used as a tool for inferring genomic instability or other forms of DNA damage (*61*−*63*).

Conclusions. In general, this study provides evidence of increased mutation rate and an increased immigration/emigration ratio (i.e., the more highly contaminated populations are now or have been in the past, more "sinklike" than the less contaminated populations) as a result of pulp mill effluent. In addition, the increased genetic diversity in the contaminated populations may be due to increased mutation rate and gene flow, and increased mutation rate may have more of an influence in PRK 89−103, while gene flow may have more of an influence in PRK 27. Note that these patterns are probably not due (at least exclusively) to a natural upstream−downstream gradient, because PRK 42 (less contaminated than the other Pigeon River sites) is upstream of PRK 27 but downstream of PRK 89−103. Also, bioindicator data suggest that there has been recovery of PRK 42 after diversion of the main river channel away from this site (*34*−*36*), and this recovery is reflected in population genetic data presented above as well.

There are several other conclusions from this study that are worthy of mention. The first is that there is a high level of gene flow in this species for the river systems in question, even though there are several potential dispersal barriers (i.e., dams). There may be two different explanations for this. First, the genetic indicators of gene flow may reflect historic processes that occurred before the dams were built. This does not preclude effects of contamination on gene flow, because the Champion Paper Mill began operations near the turn of the 20th century, while the dams were not built before 1935. Undoubtedly, the amount of contaminants that were released at this time were greater than that in the later half of that century, when environmental regulations were enacted. Thus, unrestricted gene flow was possible among populations in the Pigeon River and between the Pigeon River and other rivers within the Tennessee Valley, for at least several dozen generations, at the same time when populations of the Pigeon River were exposed to high levels of contamination. The second general conclusion is that the RAPD technique may be more sensitive to the effects of contaminant-induced mutations/genomic rearrangements than other molecular markers. Third, having population census data

that provides information on metrics that affect effective population size (population density, sex ratios, age/size structure) can provide additional information useful in interpreting genetic metrics. Fourth, genetic diversity is not always reduced as a result of contaminant exposure, especially for RAPD markers. Finally, implicating contamination as the cause of population genetic effects may require a weight of evidence approach, employing several different genetic techniques and alternative analyses along with indicators of exposure and effects such as biomarkers (*29*). This is because any genetic-based estimator of population processes or population history may be biased, so that conclusions must be made on the basis of the overall pattern of all indicators.

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Supporting Information Available

More detailed information for definitions of population genetics terms and concepts presented herein, more detailed information on the RAPD bands used, and tables of data containing comparisons of Fst, assignment test results, and θ among each pair of populations. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Larno, V.; Laroche, J.; Launey, S.; Flammarion, P.; Devaux, A. Responses of chub (*Leuciscus cephalus*) populations to chemical stress, assessed by genetic markers, DNA damage and cytochrome P4501A induction. *Ecotoxicology* **2001**, *10*, 145−158.

(2) Mulvey, M.; Newman, M. C.; Vogelbein, W. K.; Unger, M. A.; Ownby, D. R. Genetic structure and mtDNA diversity of *Fundulus heteroclitus* populations from polycyclic aromatic hydrocarbon-contaminated sites 2003. *Environ. Toxicol. Chem.* **2003**, *22*, 671−677.

(3) Roark, S. A.; Nacci, D.; Coiro, L.; Champlin, D.; Guttman, S. I. Population genetic structure of a nonmigratory estuarine fish (*Fundulus heteroclitus*) across a strong gradient of polychlorinated biphenyl contamination. *Environ. Toxicol. Chem.* **2005**, *24*, 717−725.

(4) Theodorakis, C. W.; Bickham, J. W.; Lamb, T.; Medica, P. A.; Lyne, T. B. Integration of genotoxicity and population genetic analysis in kangaroo rats (*Dipodomys merriami*) exposed to radionuclide contamination. *Environ. Toxicol. Chem.* **2001**, *20*, 317−326.

(5) Kim, S. J.; Rodriguez-Lanetty, M.; Suh, J. H.; Song, J. I. Emergent effects of heavy metal pollution at a population level, *Littorina brevicula* a study case. *Mar. Pollut. Bull.* **2003**, *46*, 74−80.

(6) Whitehead, A.; Anderson, S. L.; Kuivila, K. M.; Roach, J. L.; May, B. Genetic variation among interconnected populations of *Catostomus occidentalis*, implications for distinguishing impacts of contaminants from biogeographical structuring. *Mol. Ecol.* **2003**, *12*, 2817−2833.

(7) Staton, J. L.; Schizas, N. V.; Chandler, G. T.; Coull, B. C.; Quattro, J. M. Ecotoxicology and population genetics, The emergence of phylogeographic and evolutionary ecotoxicology. *Ecotoxicology* **2001**, *10*, 217−222.

(8) DeWolf, H.; Blust, R.; Backeljau, T. The population genetic structure of *Littorina littorea* (Mollusca, Gastropoda) along a pollution gradient in the Scheldt estuary (The Netherlands) using RAPD analysis. *Sci. Total Environ.* **2004**, *325*, 59−69.

(9) Keane, B.; Collier, M. H.; Rogstad, S. H. Pollution and genetic structure of North American populations of the common dandelion (*Taraxacum officinale*). *Environ. Monit. Assess.* **2005**, *105*, 341−357.

(10) Benton, M. J.; Malott, M. L.; Trybula, J.; Dean, D. M.; Guttman, S. I. Genetic effects of mercury contamination on aquatic snail populations, Allozyme genotypes and DNA strand breakage. *Environ. Toxicol. Chem.* **2003**, *21*, 584−589.

(11) Gillespie, R. B.; Guttman, S. I. Chemical-induced changes in the genetic structure of populations, Effects on allozymes. In *Genetics and Ecotoxicology*; Forbes, V. E., Ed.; Taylor & Francis: New York, 1999; pp 55−77.

(12) Theodorakis, C. W.; Shugart, L. R. Natural selection in contaminated habitats: A case study using RAPD genotypes. In *Genetics and Ecotoxicology*; Forbes, V. E., Ed.; Taylor & Francis: New York, 1999; pp 123−150.

(13) Krane, D. E.; Sternberg, D. C.; Burton, G. A. Randomly amplified polymorphic DNA profile-based measures of genetic diversity in crayfish correlated with environmental impacts. *Environ. Toxicol. Chem.* **1999**, *18*, 504−508.

(14) Ma, X. L.; Cowles, D. L.; Carter, R. L. Effect of pollution on genetic diversity in the bay mussel *Mytilus galloprovincialis* and the acorn barnacle *Balanus glandula*. *Mar. Environ. Res.* **2000**, *50*, 559−563.

(15) Silbiger, R. N.; Leonard, A. C.; Dimsoski, P.; Fore, S.; Guttman, S. I.; Roth, A. C.; Gordon, D. A.; Wessendarp, T.; Toth, G. P.; Smith, M. K. Use of molecular markers to study the effects of environmental impacts on genetic diversity in brown bullhead (*Ameirus nebulosus*) populations. *Environ. Toxicol. Chem.* **2001**, *20*, 2580−2587.

(16) Ross, K.; Cooper, N.; Bidwell, J. R.; Elder, J. Genetic diversity and metal tolerance of two marine species: A comparison between populations from contaminated and reference sites. *Mar. Pollut. Bull.* **2002**, *44*, 671−679.

(17) Turuspekov, Y.; Adams, R. P.; Kearney, C. M. Genetic diversity in three perennial grasses from the Semipalatinsk nuclear testing region of Kazakhstan after long-term radiation exposure. *Biochem. Syst. Ecol.* **2002**, *30*, 809−817.

(18) Keane, B.; Pelikan, S.; Toth, G. P.; Smith, M. K.; Rogstad, S. H. Genetic diversity of *Typha latifolia* (Typhaceae) and the impact of pollutants examined with tandem-repetitive DNA probes. *Am. J. Bot.* **1999**, *86*, 1226−1238.

(19) Prus-Glowacki, W.; Wojnicka-Poltorak, A.; Oleksyn, J.; Reich, P. Industrial pollutants tend to increase genetic diversity, Evidence from field-grown european scots pine populations. *Water, Air, Soil Pollut.* **1999**, *116*, 395−402.

(20) Matson, C. W.; Rodgers, B. E.; Chesser, R. K.; Baker, R. J. Genetic diversity of *Clethrionomys glareolus* populations from highly contaminated sites in the Chernobyl Region, Ukraine. *Environ. Toxicol. Chem.* **2000**, *19*, 2130−2135.

(21) Baker, R. J.; Bickham, A. M.; Bondarkov, M.; Gaschak, S. P.; Matson, C. W.; Rodgers, B. E.; Wickliffe, J. K.; Chesser, R. K. Consequences of polluted environments on population structure, the bank vole (*Clethrionomys glareolus*) at Chornobyl. *Ecotoxicology* **2001**, *10*, 211−216.

(22) Moraga, D.; Mdelgi-Lasram, E.; Romdhane, M. S.; El Abed, A.; Boutet, I.; Tanguy, A.; Auffret, M. Genetic responses to metal contamination in two clams, *Ruditapes decussatus* and *Ruditapes philippinarum*. *Mar. Environ. Res.* **2002**, *54*, 521−525.

(23) Chen, X. Y.; Li, N.; Shen, L.; Li, Y. Y. Genetic structure along a gaseous organic pollution gradient, A case study with *Poa annua* L. *Environ. Pollut.* **2003**, *124*, 449−455.

(24) Peles, J. D.; Towler, W. I.; Guttman, S. I. Population genetic structure of earthworms (*Lumbricus rubellus*) in soils contaminated by heavy metals. *Ecotoxicology* **2003**, *12*, 379−386.

(25) Li, N.; Chen, X. Y.; Shen, L.; Li, Y. Y.; Cai, Y. W. Effects of traffic pollution on the genetic structure of *Poa annua* L. populations. *J. Environ. Sci.* **2004**, *16*, 454−457.

(26) Yap, C. K.; Tan, S. G.; Ismail, A.; Omar, H. Allozyme polymorphisms and heavy metal levels in the green-lipped mussel *Perna viridis* (Linnaeus) collected from contaminated and uncontaminated sites in Malaysia. *Environ. Int.* **2004**, *30*, 39−46.

(27) Bickham, J. W.; Sandhu, S.; Hebert, P. D. N.; Chikhi, L.; Athwal, R. Effects of chemical contaminants on genetic diversity in natural populations, implications for biomonitoring and ecotoxicology. *Mutat. Res.* **2000**, *463*, 33−51.

(28) Theodorakis, C. W.; Wirgin, I. Genetic responses as population-level biomarkers of stress in aquatic ecosystems. In *Biological Indicators of Aquatic Ecosystem Health*; Adams, S. M., Ed.; American Fisheries Society: New York, 2002; pp 147−186.

(29) Theodorakis, C. W. Establishing causality between population genetic alterations and environmental contamination in aquatic organisms. *Hum. Ecol. Risk Assess.* **2003**, *9*, 37−58.

(30) Shugart, L. R.; Theodorakis, C. W.; Bickham, A. M.; Bickham, J. Genetic effects of contaminant exposure and potential impacts on animal populations. In *Handbook of Ecotoxicology*; Calow, P., Ed.; Blackwell Science: Oxford, U. K., 2002.

(31) Avise, J. C. The history and purview of phylogeography, A personal reflection. *Mol. Ecol.* **1998**, *7*, 371−379.

(32) Manel, S.; Schwartz, M. K.; Luikart, G.; Taberlet, P. Landscape genetics, combining landscape ecology and population genetics. *Trends Ecol. Evol.* **2003**, *18*, 189−197.

(33) *Environmental Fate and Effects of Pulp and Paper Mill Effluents*; Servos, M. R., Munkittrick, K. R., Carey, J. H., van, der, Kraak, G. J., Eds.; St. Lucie Press: Delray Beach, FL, 1996.
(34) Adams, S. M.; Shepard, K. L.; Greeley, M. S.; Jimenez, B. D.; Ryon, M. G. The Use of Bioindicators for Assessing the Effects of Pollutant Stress on Fish. *Mar. Environ. Res.* **1989**, *28*, 459−464.
(35) Adams, S. M.; Crumby, W. D.; Greeley, M. S.; Shugart, L. R. Responses of fish populations and communities to pulp mill effluents, A holistic assessment. *Ecotoxicol. Environ. Saf.* **1992**, *24*, 347−360.
(36) Adams, S. M.; Ham, K. D.; Greeley, M. S.; LeHew, R. F.; Hinton, D. E.; Saylor, C. F. Downstream gradients in bioindicator responses, Point source contaminant effects on fish health. *Can. J. Fish. Aquat. Sci.* **1996**, *53*, 2177−2187.
(37) Nadig, S. G.; Lee, K. L.; Adams, S. M. Evaluating alterations of genetic diversity in sunfish populations exposed to contaminants using RAPD assay. *Aquat. Toxicol.* **1998**, *43*, 163−178.
(38) Theodorakis, C. W.; Shugart, L. R. Genetic ecotoxicology, II. Population genetic structure in radionuclide-contaminated mosquitofish (*Gambusia affinis*). *Ecotoxicology* **1997**, *6*, 335−354.
(39) Lynch, M. The similarity index and DNA fingerprinting. *Mol. Biol. Evol.* **1990**, *7*, 478−489.
(40) Hartl, D. L.; Clark, A. G. *Principles of Population Genetics*, 3rd ed.; Sinauer Associates: Sunderland, MA, 1996.
(41) Black, W. C., IV *RAPDFST—A FORTRAN Program to Estimate F_{ST} and Effective Migration Rates among Subpopulations Using RAPD-PCR Files*; Colorado State University: Fort Collins, CO, 1997.
(42) Schneider, S.; Roessli, D.; Excoffier, L. *Arlequin, A Software for Population Genetics Data Analysis*, version 2.000; Genetics and Biometry Lab, Department of Anthropology, University of Geneva: Geneva, Switzerland, 2000.
(43) Black, W. C., IV, Antolin, M. *FORTRAN Programs for the Analysis of RAPD-PCR Markers, RAPDDIST*; Colorado State University: Fort Collins, CO, 1997.
(44) Black, W. C. IV *RAPDPLOT*, version 2.4; Colorado State University: Fort Collins, CO, 1996.
(45) Chen, J. Z.; Hebert, P. D. N. Intraindividual sequence diversity and a hierarchical approach to the study of mitochondrial DNA mutations. *Mutat. Res.* **1999**, *434*, 205−217.
(46) Paetkau, D.; Calvert, W.; Sterling, I.; Strobeck, C. Microsatellite analysis of population structure in Canadian polar bears. *Mol. Ecol.* **1995**, *4*, 347−354.
(47) Brzustowski, J. DOH assignment test calculator, 2002. http://www2.biology.ualberta.ca/jbrzusto/Doh.php.
(48) Beerli, P.; Felsenstein, J. Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4563−4568.
(49) Fu, Y. X. A phylogenetic estimator of effective population size or mutation rate. *Genetics* **1994**, *136*, 685−692.
(50) Frankham, R.; Ballou, J. D.; Briscoe, D. A. *Introduction to Conservation Genetics*; Cambridge University Press: New York, 2002.
(51) Neigel, J. E. Estimation of effective population size and migration parameters from genetic data. In *Molecular Genetic Approaches in Conservation*; Smith, T. B., Wayne, R. K., Eds.; Oxford University Press: New York, 1996; pp 329−346.
(52) Schwartz, M. K.; Tallmon, D. A.; Luikart, G. Using genetics to estimate the size of wild populations: Many methods, much potential, uncertain utility. *Anim. Conserv.* **1999**, *2*, 321−323.
(53) Isabel, N.; Beaulieu, J.; Thériault, P.; Bousquet, J. Direct evidence for biased gene diversity estimates from dominant random amplified polymorphic DNA (RAPD) fingerprints. *Mol. Ecol.* **1999**, *8*, 477−483.
(54) Lougheed, S. C.; Gibbs, H. L.; Prior, K. A.; Weatherhead, P. J. A comparison of RAPD versus microsatellite DNA markers in population studies of the Massasauga rattlesnake. *J. Hered.* **2000**, *91*, 458−463.
(55) Hollingsworth, P. M.; Ennos, R. A. Neighbour joining trees, dominant markers and population genetic structure. *Heredity* **2004**, *92*, 490−498.
(56) Mengoni, A.; Barabesi, C.; Gonnelli, C.; Galardi, F.; Gabbrielli, R.; Bazzicalupo, M. Genetic diversity of heavy metal-tolerant populations in *Silene paradoxa* L. (Caryophyllaceae), A chloroplast microsatellite analysis. *Mol. Ecol.* **2001**, *10*, 1909−1916.
(57) Slatkin, M.; Hudson, R. R. Pairwise comparisons fo mitochondrial DNA sequences in stable and exponentially grown populations. *Genetics* **1991**, *129*, 555−562.
(58) Good, S. V.; Williams, D. F.; Ralls, K.; Fleischer, R. C. Population structure of *Dipodomys ingens* (Heteromyidae), The role of spatial heterogeneity in maintaining genetic diversity. *Evolution* **1997**, *44*, 1296−1310.
(59) Blevins, R. D. 2,3,7,8-Tetrachlorodibenzodioxin in fish from the pigeon river of Eastern Tennessee, USA: Its toxicity and mutagenicity as revealed by the Ames Salmonella assay. *Arch. Environ. Toxicol. Chem.* **1991**, *20*, 366−370.
(60) Lynch, M.; Conery, J.; Bürger, R. Mutational meltdowns in sexual populations. *Evolution* **1995**, *49*, 1067−1080.
(61) DeWolf, H.; Blust, R.; Backeljau, T. The use of RAPD in ecotoxicology. *Mutat. Res.* **2004**, *566*, 249−262.
(62) Atienzar, F.; Child, P.; Evenden, A.; Jha, A.; Savva, D.; Walker, C.; Depledge, M.; Goksoeyr, A. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. *Mar. Environ. Res.* **1998**, *46*, 331−335.
(63) Savva, D. Use of DNA fingerprinting to detect genotoxic effects. *Ecotoxicol. Environ. Saf.* **1998**, *41*, 103−106.
(64) Templeton, A. R. Using haplotype trees for phylogeographic and species inference in fish populations. *Environ. Biol. Fish.* **1995**, *69*, 7−20.

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