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# Establishing Causality between Population Genetic Alterations and Environmental Contamination in Aquatic Organisms

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## ABSTRACT

Contaminant-induced alterations in genetic diversity or allele/genotype frequencies can occur via genetic bottlenecks, selection, or increased mutation rate, and may affect population growth, sustainability, and adaptability. Determination of causality of genetic effects requires demonstration of some or all of the following criteria: (1) *Strength of association*: use of multiple reference and contaminated populations, and demonstration of effects that cannot otherwise be explained by evolutionary theory; (2) *Consistency of association*: effects corroborated by other studies, in other species, or with multiple genetic markers; (3) *Specificity of association*: concordance of genetic effects with exposure/effect bioindicators, genotype-dependant fitness and biomarkers, and consideration of confounding factors; (4) *Temporality of effects*: use of phylogenetics and analysis of genetic diversity using different methodologies to differentiate historical vs. recent events; (5) *Biological gradients:* sampling sites that are known to have differing levels of contamination; (6) *Experimental evidence*: exposure of small populations to contaminants in laboratories, mesocosms, or *in situ* cages, or measurement of genotype-dependant biomarkers; (7) *Biological plausibility:* existence of contaminants at levels great enough to affect fitness, recruitment, or mutation rates, or a demonstrated mechanism for selection. Application of these criteria to population genetic studies is illustrated by case studies involving RAPD analysis of mosquitofish populations.

**Key Words**: population genetics, environmental contamination, causality, ecoepidemiology, evolutionary toxicology.

## INTRODUCTION

Genetic responses of aquatic organisms to environmental contamination include both genotoxic and population genetic effects. Genotoxic responses involve direct interaction of genotoxic chemicals, UV, or ionizing radiation with the DNA mol-

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ecule, occur at the level of the individual, and — unless they involve genotoxicant-induced germ-line mutations — are not multigenerational. These effects originate as DNA damage, and may be ultimately manifested as mutagenesis, carcinogenesis, and teratogenesis. Unlike genotoxic effects, population genetic responses are multigenerational (*i.e.*, they may both require multiple generations of exposure to be manifested, and may persist for generations), are manifested at the population level, and are not necessarily due to interactions of chemical or physical agents with the DNA. Detailed discussions of population genetic effects of pollution and their ecological relevance have been published elsewhere (Mulvey and Diamond 1991; Bickham and Smolen 1994; Guttman 1994; Gillespie and Guttman 1998; Theodorakis and Shugart 1998a; Bickham *et al.* 2000; Theodorakis 2001; Shugart *et al.* 2002; Theodorakis and Wirgin 2002), and therefore are not discussed at length here. Rather, this paper focuses on the application of ecoepidemiological approaches, and in particular criteria for establishing causality (Adams 2003) in studies of population genetic responses to xenobiotic stress. However, a brief summary of population genetic responses is warranted.

## TYPES OF POPULATION GENETIC RESPONSES AND THEIR SIGNIFICANCE

Contaminant-induced population genetic perturbations may be reflected in changes in relative amount of genetic diversity and allele or genotype frequencies (the study of such effects has been termed "evolutionary toxicology" (Bickham and Smolen 1994). Evolutionary toxicology is relevant to ecological risk assessments for two reasons. First, alterations in population genetic parameters may be sensitive or early warning bioindicators of other effects such as loss of species, changes in community structure, and alterations of dispersal recruitment, population growth or population dynamics (Fore *et al.* 1995a; Theodorakis and Shugart 1998a; Bickham and Smolen 1994). Second, anthropogenic changes in genetic diversity may affect the growth, evolutionary plasticity, sustainability, and probability of extinction of populations (Bickham *et al.* 2000; Theodorakis and Wirgin 2002).

The two most widely recognized effects of environmental contamination on population genetic structure are genetic bottlenecks and contaminant-induced selection. Genetic bottlenecks can reduce population genetic diversity via increased mortality, reduced reproductive output or recruitment (both from within and without the population), or selection for contaminant-resistant genotypes. Contaminant-induced selection may occur if individuals with certain genotypes are more susceptible to contaminant exposure than other individuals. In addition, polluted habitats are often highly modified, and habitat alteration/destruction may have additive or synergistic effects on population genetics when combined with contaminant-induced bottlenecks and selection.

If populations are exposed to mutagenic chemicals, then increased mutation rates may be another mechanism whereby population genetic structure is altered by contaminant exposure. Increased mutation rates may affect average fitness of the population through increased genetic load (accumulation of deleterious mutations). This affects small populations to a greater degree than large populations. Decreased fitness due to increased genetic load could then lead to reduced popu-

lation size, resulting in increased inbreeding and fixation of deleterious alleles, causing additional reductions of fitness, etc. Thus, such populations may spiral towards extinction in a process known as "mutational meltdown" (Gabriel and Bürger 1994).

### Population Genetics and Ecological Risk Assessment

There are many factors that can contribute to anthropogenic changes in population genetic parameters. These include acute or chronic toxicity, alterations in behavior, modification of community dynamics (*e.g.*, competition, predatory/prey interactions), changes in ecosystem productivity and alteration of trophic structure. Hence, population genetic effects may be emergent properties of perturbed systems, rather than direct effects of the toxicants themselves. This would obscure the relative contribution of each of the factors listed above to alterations in genetic diversity. Evolutionary history and a myriad of environmental variables may also affect population genetic diversity, so any two populations would be expected to be genetically different irrespective of xenobiotic exposure. Therefore, simply comparing a contaminated and a noncontaminated population may not provide meaningful insight into population genetic effects of contamination.

A more robust tactic would entail a weight of evidence approach, incorporating many components of ecoepidemiology in order to discriminate between natural and anthropogenic effectors of genetic diversity. Because of the unique nature of population genetic responses to pollution, some of the seven causality criteria outlined by Adams (2003) are directly applicable to evolutionary toxicology, while others may need to be modified to be compatible with this discipline. Thus, the objectives of this paper are first to discuss how causality criteria can be applied to or modified for population genetic studies, and second to present a case study illustrating application of these criteria to population genetic studies. The seven causality criteria outlined by Adams (2003) include (1) strength of association, (2) consistency of association, (3) specificity of association, (4) time order or temporality, (5) biological gradient, (6) experimental evidence, and (7) plausibility.

## CAUSALITY CRITERIA

### Strength of Association

This criterion requires demonstration that the cause and effect coincide or that the endpoint in question is sensitive to pollutant stress (Luoma *et al.* 2001). For population genetic studies, this would entail (1) comparing the observed and expected differences between populations, (2) determining effects of population subdivision, and (3) testing the alternative hypothesis that genetic differences between populations are due to neutral variation between populations.

### Observed vs. Expected Differences

Before inferences can be made about effects of pollution on population genetics, the background level of diversity between populations must be estimated. This would require characterization of genetic diversity in multiple reference sites, and if possible, multiple contaminated sites. Such an experimental design would allow

the investigator to determine if the differences between the contaminated and reference sites are greater than expected between any two reference sites. Bootstrapping (repeated subsampling with replacement) could also be used to test for heterogeneity in genetic variance between and within populations, using techniques analogous to analysis of variance (Excoffier *et al.* 1992).

Additionally, observed patterns of genetic diversity could be compared with those expected on the basis of evolutionary theory, which takes into account such factors as gene flow, common ancestry, and geographic distribution of populations. Significant deviations from results expected from evolutionary models would provide evidence of contaminant-induced effects. For example, Fore *et al.* (1995a) sampled fish from a site contaminated by an industrial effluent, as well as from less contaminated sites upstream and downstream of this point source. They found that the upstream and downstream populations were more genetically similar to each other than to the contaminated population located between them. These findings are contradictory from what would be predicted from stepping stone or isolation by distance models, which assume genetic similarity is inversely proportional to geographic distance between populations (Hartl and Clark 1997). Therefore, careful choice of geographic locations of reference sites could allow testing to determine if patterns of genetic diversity and relatedness among reference and polluted sites conform to those predicted by models of gene flow or common ancestry.

### Population Subdivision

When examining the effects of pollution on population genetic structure, it is advisable to determine if genetic subdivision influences apparent differences between populations. For example, Woodward *et al.* (1996) examined heterozygosity in benthic chironomids, and found that differences in heterozygosity between contaminated and reference sites may have been due to heterogeneous distribution of allele frequencies within the population rather than overt toxic effects *per se.* However, an increase in population subdivision could also be caused by anthropogenic disturbance, due to physical habitat fragmentation or highly heterogeneous contaminant concentrations (as is often the case in contaminated sediments).

### Neutral Genetic Variation

Two alternative explanations for allele frequency differences between contaminated and reference populations are contaminant-induced selection and neutral genetic variation between populations. Several methods have been developed for testing the neutrality hypothesis. For example, the Ewans-Waterson test uses bootstrap sampling of alleles, while the test developed by Taijima (1989a) uses DNA sequence information, estimated mutation rates, and effective population sizes to test for neutral vs. non-neutral evolution (Tajima 1989a). By itself, rejection of the neutrality hypothesis does not indicate that selection has occurred, but this can be an important contribution to establishing strength of association and weight of evidence.

### Phylogenetic Relationships

The discipline of phylogenetics examines evolutionary relationships among populations, alleles, or haplotypes. These relationships are usually represented visually by

a phylogenetic tree (Figure 1). Each branch on a phylogenetic tree is called a clade, and represents a group of evolutionarily related individuals. Phylogenetics can be important for demonstrating strength of association by assisting in experimental design. For example, when choosing reference sites, it would be best not to develop a sampling scheme where a contaminated site is in one clade and all reference sites are in a different clade. Otherwise, differences between contaminated and reference sites may be due to evolutionary history rather than contamination. It would be better to select reference sites that are in the same clade as the contaminated site, or choose reference and contaminated sites that are homogenously distributed among clades.

Phylogenies can also reveal contaminant effects via patterns of gene flow between populations. For example, many contaminated populations may be ecological "sinks", that is, population growth and production is low, so that the rate of immigration from other populations is far less than the rate of emigration and dispersal to other populations. For these populations, recruitment from within the population may be insufficient to maintain a viable population, and the population may be sustained only by immigration. In this case, the genetic similarity between contaminated and reference populations should be greater than the similarity among reference populations. This approach can be further refined by incorporation of biogeography of alleles or haplotypes into the phylogenetic analysis (*i.e.*, "phylogeography"). Phylogeography can be used to determine the direction of dispersal and gene flow (Slatkin and Maddison 1989) — for example, dispersal (and resultant gene flow) from reference populations into contaminated sites rather than *vice versa*.

**Consistency of Association**

The consistency of association criterion involves demonstrating that observed effects are corroborated by other investigators and/or at other places or times. For population genetic studies, this could also include demonstrating similar effects in other species from the same locations, and parallel responses in studies using different genetic markers or multiple loci. A good illustration of this concept is provided by the work of Oris, Guttman and colleagues (Miami University, Oxford, OH) and Mulvey, Newman, and co-workers (College of William and Mary, Virginia Institute of Marine Science, Gloucester Point, VA). These investigators have used allozyme analysis for many years to examine effects of pollutants on population genetic parameters. In several field studies, similar responses were found for the same species of fish collected from different contaminated sites, and for different fish collected from the same field site (Gillespie and Guttman 1988; Heagler *et al.* 1993; Fore *et al.* 1995a,b). Laboratory or mesocosm studies also revealed parallel trends in same organisms exposed to different contaminants, and in different organisms exposed to the same contaminants (Changon and Guttman 1989a; Diamond *et al.* 1989; Mulvey *et al.* 1995; Schlueter *et al.* 1995; Tatara *et al.* 1999; Duan *et al.* 2000a,b; Schlueter *et al.* 2000). In fact, these studies have found that the same loci (*e.g.*, phosphoglucomutase, glucose phosphate isomerase) are often associated with resistance to a variety of pollutants in diverse organisms.

**Specificity of Association**

This criterion entails differentiating between stressor effects and environmental variability (Luoma *et al.* 2001; Suter 1993). In population genetic studies, this

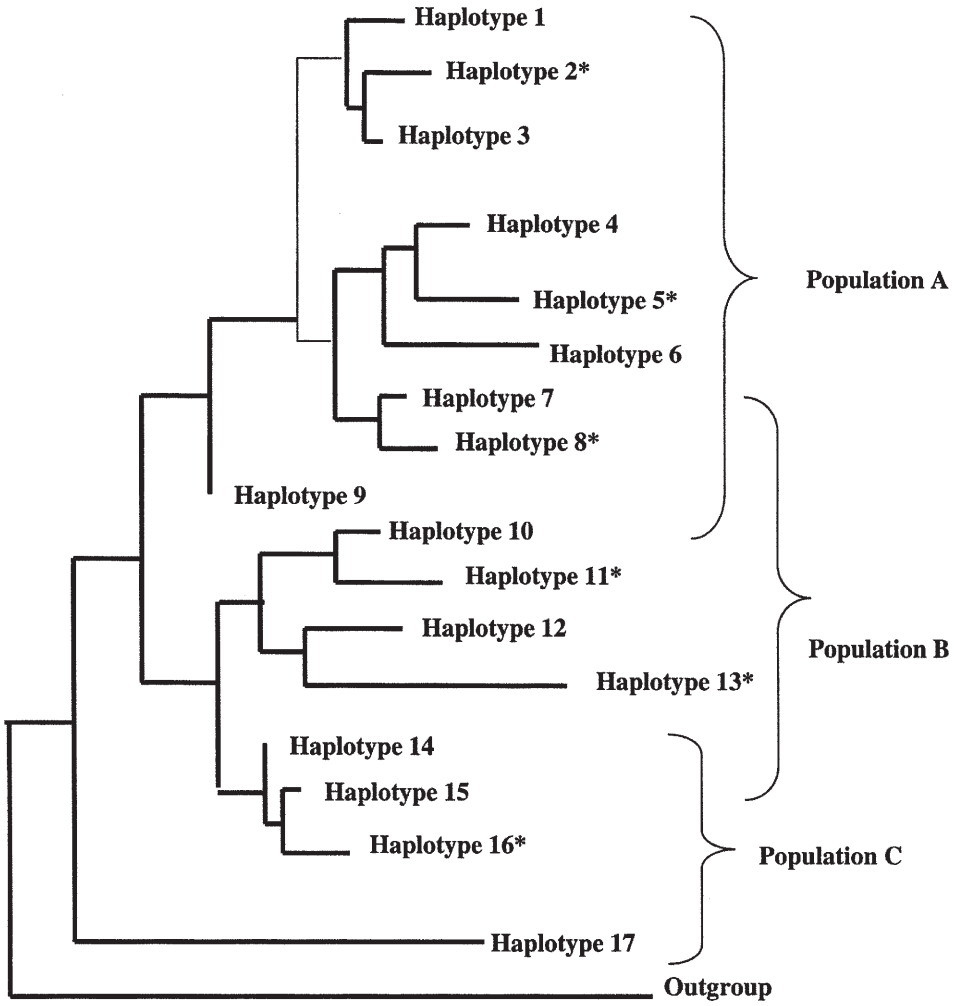


**Figure 1. An example of a phylogenetic tree of 17 mitochondrial haplotypes from three hypothetical populations.** Several things are inferred from this tree: (1) some haplotypes are found in more than one population, suggesting that there has been gene flow between populations; (2) the age of the haplotypes can be inferred from the position on the tree: *e.g.*, haplotypes 1 & 2 are terminal branch haplotypes, while haplotype 17 is deeply rooted in the tree, so 17 is much older than 1 &2; (3) haplotype 2 is more similar to 3 than it is to 1; 4) haplotype 9 is probably the ancestral haplotype to all other haplotypes unique to population 1; 5) haplotypes 15 and 16 share a common ancestor, and 9 is more similar to this common ancestor than 16 (16 is more "derived"). The "outgroup" is a closely related species used to "root" the tree. Terminal branch haplotypes are indicated by an asterisk (*).

criterion involves inter- and intrapopulation comparisons of population genetic and bioindicator responses, as well as experimental designs and statistical analyses that identify or minimize the effect of confounding environmental variation.

### Interpopulation Comparisons

Bioindicators of exposure include chemical body burden data and biomarkers of exposure, while bioindicators of effect include biomarkers of effect, population or community level parameters such as population declines and index of biotic integrity (IBI) scores, and gross injuries including tumors, lesions, deformities, and fish or bird kills (Suter 1993). In order to demonstrate specificity of association, patterns of contaminant-induced genetic alterations should parallel trends in bioindicator responses. For example, Krane *et al.* (1999) sampled several Ohio rivers contaminated with urban effluent, and found that genetic diversity in crayfish populations was correlated with fish community IBI scores from the same sampling locations. In another study, Benton *et al.* (2001) found both altered genotype frequencies and increased DNA damage in aquatic snail populations stressed by mercury contamination.

### Intrapopulation Comparisons

Many studies have found that, for some loci, one allele is more common in contaminated than in reference populations (for the sake of discussion, these will be referred to as "contaminant-resistant alleles"). In this situation, fitness components and bioindicators of deleterious effect can be compared between individuals with different genotypes. If the allele frequencies are truly due contaminant-induced selection, then fitness components (*e.g.*, reproductive success, survival, growth, and development) and the bioindicators of deleterious effects (*e.g.,* biomarkers, lesions, tumors and deformities) should vary between individuals in a genotype-dependant manner. In some salutations there may be multiple loci that contribute to genetic differences between contaminated and reference populations, so that there are multiple contaminant-resistant alleles. In this case, there may be a correlation between the magnitude of fitness or bioindicator responses and the number of contamination resistant alleles per individual.

These fitness components and bioindicators of contaminant effects should be genotype dependant in contaminated populations, but not in reference populations. Such an association in the absence of contamination would indicate that this is a general phenomenon, and not related to contamination *per se*. Conversely, if individuals with contaminant-resistant genotypes have lower fitness than contaminant-sensitive genotypes in noncontaminated populations, this may indicate a cost of adaptation or fitness tradeoff (see Theodorakis and Shugart 1998a for discussion).

### Experimental Design

To help address the specificity of association criterion, the experimental design should be such that the reference sites are as similar as possible to the study site, and/or the environmental conditions of the reference sites bracket those of the contaminated site. For example, if the contaminated site is a first-order stream with

a gravel substrate, then similar first-order streams with gravel substrates should be chosen as reference sites. However, there would still be some degree of difference in physiochemical parameters (*e.g.*, stream flow velocity and water temperature) between such streams. Therefore, the reference sites should be chosen to be slightly faster/colder and slightly slower/warmer than the study site. If the study site is a second-order stream and, for logistical reasons, it is not possible to use second-order streams as reference sites, then first- and third-order streams might be chosen as reference sites (*e.g.*, as opposed to choosing all first order streams as reference sites).

The identification of such environmental variables that affect population genetic structure is also important for differentiating between natural and contaminant effects on populations. For instance, multivariate techniques could be used to discern which environmental variables contribute the most to genetic differences among reference sites. It could then be determined if the genetic differences between contaminated and reference sites conform to expectations based on these variables. Environmental contamination could also be included as one of the environmental variables in the analysis, and multivariate statistics could be used to determine if contamination was one of the major contributors to genetic differences between populations.

### Time Order or Temporality

This criterion requires that the alleged stressor must precede the observed effect, and that the effect must decrease when the stressor is mitigated. Because population genetic effects may take several generations to manifest themselves, this may be difficult for species with relatively long generation times. For species with shorter generation times, this criterion can be satisfied by monitoring reversal of genetic effects in remediated sites, and by using phylogenetics to make inferences about recent vs. historical evolutionary events.

### Reversal of Effects

Temporality of expression for contaminant effects on genetic diversity is influenced by both intrinsic (*e.g.*, population size) and extrinsic (*e.g.*, immigration from other populations) factors. The rate of reversal of contaminant effects is dependant on the level of gene flow between populations and the generation time of the organism in question. Reversal of population genetic alterations may also be rapid if individuals with contaminant-resistant genotypes were at a selective disadvantage in noncontaminated environments.

### Recent vs. Historical Events

Population genetic effects are unique among the responses to pollution in that patterns of genetic diversity are influenced by the history of the population. This is germane to the temporality criterion because different measurements of genetic variability can distinguish between recent and historical events (anthropogenic effectors would be among the more recent influences on population genetic structure). For example, two measures of genetic diversity include the number of alleles in a population and average heterozygosity of all loci. The number of alleles is more indicative of current population size, whereas average heterozygosity is more reflec-

tive of historic population size (Tajima 1989b). Another population genetic parameter, θ, can be calculated from DNA sequence data. This parameter is dependent on effective population size (the number of individuals in the population that contribute to the gene pool) and the mutation rate. There are two different methods of calculating , denoted $\theta_s$ and $\theta_k$ (the details of this are beyond the scope of this paper, but the reader is referred to Tajima 1989b). The $\theta_s$ is more reflective of current or recent population size, while $\theta_k$ is more influenced by historic population size, so the relative ratio of $\theta_s/\theta_k$ can be used to make inferences about recent changes in population size, assuming that mutation rate remains relatively constant over time (which may not be the case for populations exposed to mutagens). Also, markers such as microsatellites and mitochondrial loci may evolve more quickly than allozymes loci, so that changes in microsatellite or mitochondrial diversity may be more reflective of recent events than allozyme diversity. Therefore, comparative analysis of multiple genetic markers or different measures of genetic diversity can help discriminate between recent and historical changes in population genetic parameters.

This discrimination can also be accomplished through the analysis of phylogenetic relationships, because the phylogenetic tree may provide information as to the relative age of alleles or haplotypes. The younger haplotypes or alleles should be distributed among the terminal branches, while the older ones would be more deeply "rooted" in the tree (Figure 1). Chen and Herbet (1999a,b) used this phenomenon to determine if pollution exposure resulted in an increased mutation rate in fish. They argued that newly arisen mutant mitochondrial haplotypes should be located at the terminal branches of a haplotype phylogeny, so that an increased mutation rate would be reflected by an increased frequency of these "terminal branch haplotypes". Comparison of terminal vs. deeply rooted branches can also help in distinguishing between recent and historical changes in genetic diversity, population size, or dispersal events. In addition, phylogeographic approaches such as nested clade analysis (Templeton 1998) can be used to distinguish between effects of recent gene flow and evolutionary history on population genetic structure.

Phylogenetic/phylogeographic relationships could also reflect population density and provide insight as to whether a population is growing, expanding, or declining (Good *et al.* 1997). For instance, rapidly growing populations should have a higher ratio of emigrants/immigrants and a higher number of recently derived alleles than populations that are declining. Comparison of the phylogenies of contaminated and reference populations could thus provide evidence of contaminant effects on population growth and density.

**Biological Gradient**

Determination of a biological gradient in genetic diversity and genotype frequencies is fairly straightforward and similar to determination of gradients for other types of contaminant responses, so it will not be discussed at length here. However, if there is a gradient in pollution that does not correspond to a gradient in habitat modification, then this could help distinguish between contaminant stress and other types of anthropogenic disturbance as modifiers of population genetic diversity. Data from biological gradients can be gathered by sampling sites that are

various distances from a known source of contamination, or by sampling various locations that are known to have differing levels of contamination. Both Fore *et al.* (1995a,b) and Nadig *et al.* (1998) used this approach to show that the populations of fish collected below an effluent were genetically divergent from populations upstream from the effluent or from non-contaminated streams, but that the magnitude of this divergence depended on the distance of the contaminated population from the point source.

**Experimental Evidence**

Experimental evidence of population genetic effects can encompass laboratory exposures, *in situ* caging studies, microcosms/mesocosms, or field applications of chemicals. In order to establish causality, similar responses should be seen in field and laboratory exposed populations. For example, Street and Montagna (1996) found that mitochondrial DNA diversity in benthic marine copepods was reduced around oil drilling platforms. Follow-up experiments found similar responses in laboratory-reared populations exposed to polycyclic aromatic hydrocarbons (Street *et al.* 1998). These types of experimental manipulations are limited to organisms with relatively small body size or short generation times.

Experimental evidence of contaminant-induced selection can also involve individual-level responses in experimentally exposed individuals. These include genotype-specific fitness parameters (reproduction, development, survival, growth, bioenergetics) or contaminant-indicative bioindicators. In one such study, Gillespie and Guttman (1988) found allele frequency differences between metal-contaminated and noncontaminated populations of stoneroller minnows (*Campostoma anomalum*). These findings were corroborated by genotype-dependant differences in survival for individuals exposed to copper in the laboratory (Changon and Guttman 1989a). This type of experiment can be viewed as *a priori* (looking for genotype-dependant responses after differences have been found in field populations), but *a posteriori* experiments have also been conducted. For example, Heagler *et al.* (1993) exposed mosquitofish (*Gambusia holbrooki*) to metals in the laboratory and found genotype-dependant differences in time-to-death. They then sampled metal-contaminated populations to look for comparable responses in the field. Similarly, Mulvey *et al.* (1995) found an increased frequency of the same alleles, and differential fecundity between genotypes, in microcosm populations of mosquitofish exposed to the same metals. A different type of *a posteriori* experiment would be to select for contaminant-resistant and nonresistant individuals without *a priori* knowledge of genotype, and then determine if genotypic differences between selected and nonselected laboratory populations correspond to differences seen between contaminated and reference populations in the field.

Phylogenetic relationships may also be used to provide experimental evidence of adaptation. Using this approach, organisms could be collected from various non-contaminated populations, and then could be exposed to contaminants in the laboratory or caged at contaminated sites. Genetic distances could then be determined between the samples before and after contaminant exposure. For instance, Duan *et al.* (2000a) exposed amphipods (*Hyella azteca*) from different genetic stocks to metals or low pH, and found that the genetic distance between the survivors of

the exposure was less than the genetic distance between the original samples (*i.e.*, contaminant exposure made the populations more genetically similar).

Although not part of empirical experimentation, population genetic model simulation experiments can also be used to provide weight of evidence. Population genetic models have been used for both generating and testing hypotheses about natural populations. Their usefulness in establishing causality would include incidences where general patterns seen in model output coincide with observed differences between populations, for instance, in testing to see whether bottlenecks, selection, or increased mutation rate could explain observed differences between contaminated and reference populations. Such approaches have been used to investigate effect of selection (Newman and Jagoe 1998; Groeters and Tabashnik 2000) and increased mutation rates (Cronin and Bickham 1998) on population genetic structure in fish.

### Plausibility

This criterion dictates that there must be a biologically plausible mechanism whereby the stressors can induce the effects. In terms of population genetics, this would require that contaminants exist (or have existed in the past) at levels great enough to affect survival, reproductive success, recruitment, or mutation rates. If there is a hypothesis that population genetic effects are due to selection at functional loci, then there must be a mechanism for such selection. For example, various investigators have examined respiratory enzyme loci in fish, and have found differences in allele frequencies between metal-contaminated and reference populations (either natural or experimental populations). Such differences in allele frequencies were concordant with genotype-dependant survival in metal-exposed fish, genotype-specific enzyme inhibition by metals *in vitro* (Changon and Guttman 1989a,b; Kramer *et al.* 1992) and genotype-specific standard metabolic rate of metal-exposed fish *in vivo* (Kramer and Newman 1994).

## CASE HISTORY — EFFECTS OF RADIONUCLIDES ON MOSQUITOFISH

In a series of papers, Theodorakis and colleagues (Theodorakis and Shugart 1997, 1998b; Theodorakis *et al.* 1998, 1999) studied the effects of radionuclide contamination on population genetics of western mosquitofish (*Gambusia affinis*) in and around the Oak Ridge National Laboratory (ORNL) in Oak Ridge, TN. These studies have been summarized in detail elsewhere (Theodorakis 2001; Theodorakis and Shugart 1998a) and will be briefly discussed to illustrate how to apply the causality criteria to population genetic studies.

These studies focused on two populations contaminated with radionuclides - Pond 3513 and White Oak Lake — and two non-contaminated populations — Crystal Springs and Wolf Creek (Figure 2). Prior to 1977, Pond 3513 did not contain any fish. At that time, this settling basin was colonized with an intentional introduction of about 250 mosquitofish from Crystal Springs. In 1993, these populations were sampled and the genetic structure of these mosquitofish populations was analyzed using the randomly amplified polymorphic DNA (RAPD) technique. This technique uses PCR to amplify DNA fragments of various sizes from DNA samples, and, when separated by agarose gel electrophoresis, they produce a series of bands

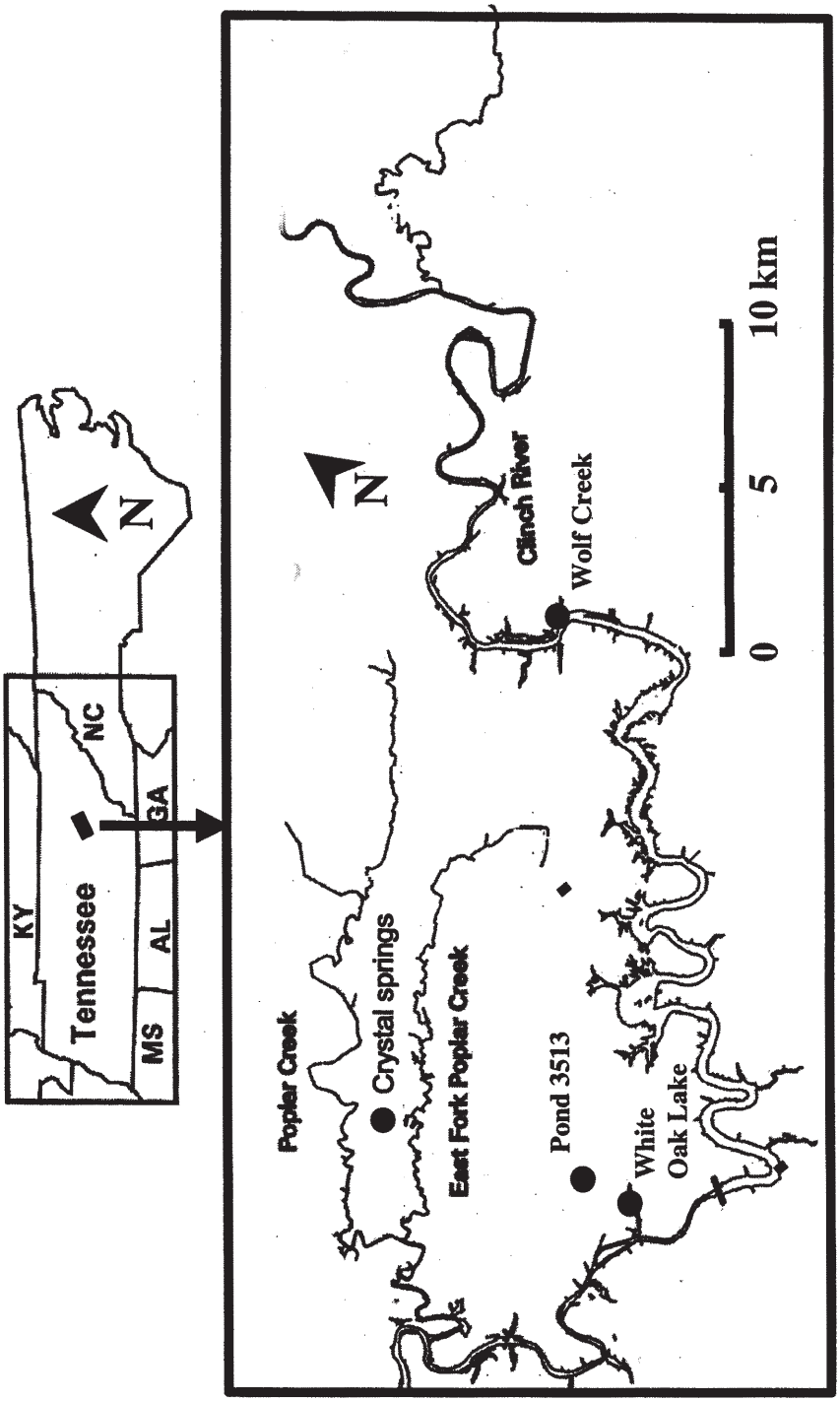


**Figure 2.** Sampling sites in and around Oak Ridge National Laboratory, from which mosquitofish (*Gambusia affinis*) were collected for population genetic analysis.

similar to DNA fingerprinting (Figure 3). RAPD analysis of these populations revealed the following: (1) the genetic diversity at the two contaminated sites was higher than in the two reference sites; (2) the DNA banding patterns in fish from the two contaminated sites were more closely related to each other than to DNA banding patterns in fish from either reference site; and (3) there were several RAPD bands that were present at a higher frequency in the contaminated than in the reference populations (for the sake of discussion, these bands will be referred to as "contaminant-indicative bands, group 1" or CIB1). Other bands were present at lower frequency in the contaminated populations than in the reference populations ("contaminant-indicative bands, group 2" [CIB2]). These data suggest that there may have been a selective advantage for fish with CIB1 and a selective disadvantage for fish with CIB2.

Allozyme studies were also performed on these same populations, and the following results were obtained: (1) the contaminated populations had higher levels of heterozygosity and percent polymorphisms than the noncontaminated sites; (2) for the nucleoside phosphorylase (NP) locus, there were two alleles (herein designated allele 1 and allele 2). Allele 1 was present at a higher frequency in the contaminated populations than in the reference populations (suggesting that there might be a selective advantage for this allele). There were no such patterns for any other allele; (3) for allozyme analysis, Pond 3513 was most closely related to Crystal Springs, and Wolf Creek was most closely related to White Oak Lake. The following discussion illustrates how all seven of the causality criteria can be applied to these data.

### Strength of Association

The fact that both contaminated sites had higher genetic diversity than both reference sites is consistent with the hypothesis that radionuclide exposure influenced population genetic structure in these mosquitofish populations. This is also true for the frequency of CIBs and NP allele 1. In addition, the fact that Pond 3513 was more closely related to White Oak Lake (in terms of RAPD analysis) was opposite of what would be expected based on evolutionary theory; because the Pond 3513 population originated from Crystal Springs, it is expected that these two populations would be the most closely related.

### Consistency of Association

In another study (Theodorakis *et al.* 1998), eastern mosquitofish (*G. holbrooki*) were collected from two radionuclide-contaminated ponds (Pond A and Pond B) and two reference populations (Risher Pond and Fire Pond) on or near the U.S. Department of Energy's Savannah River Site. These populations were examined using the same RAPD markers as those used in the *G. affinis* study. It was found that four of the CIB1 markers identified in *G. affinis* were present in the Ponds A and B populations at a higher frequency than in the Risher or Fire Pond populations. Also, two of the CIB2 bands were present at a lower frequency in Ponds A & B than in Fire or Risher Ponds. Southern blot analysis indicated that these DNA markers were homologous between the two species.

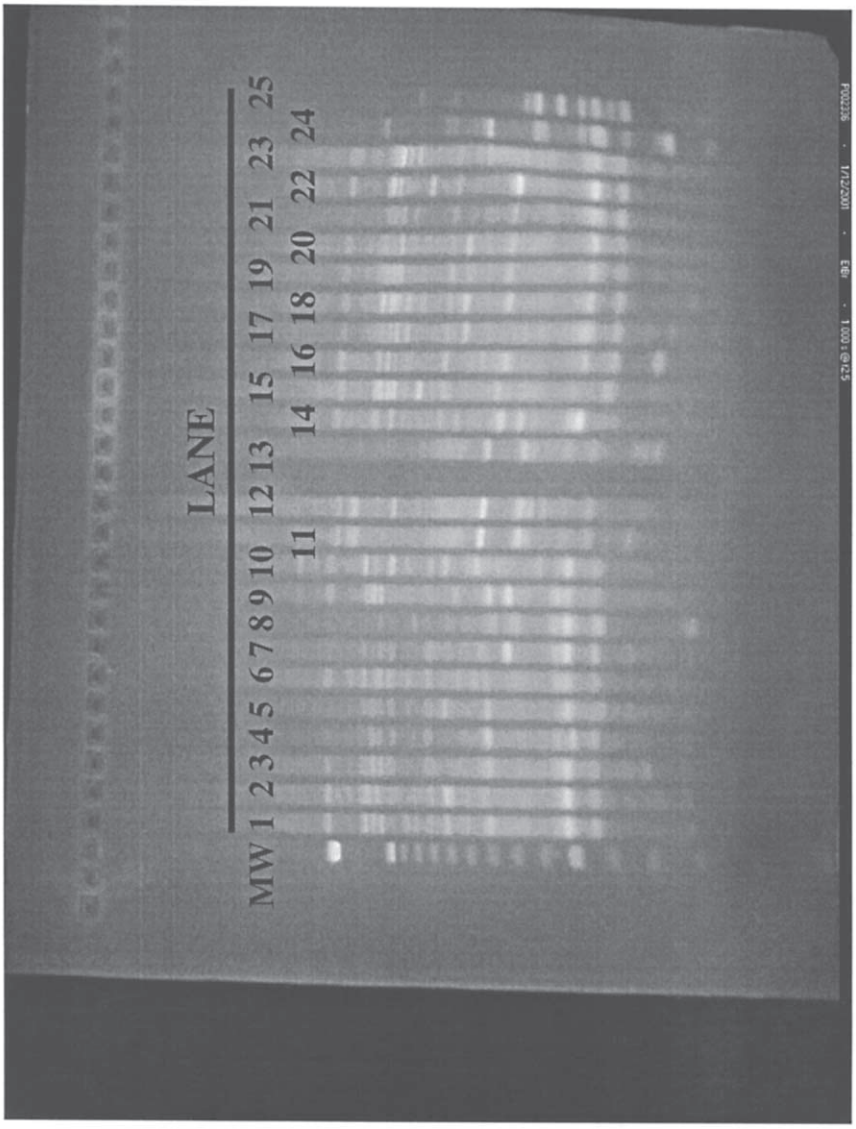


**Figure 3. Picture of a RAPD gel.** Each lane of bands represents a separate individual. MW= molecular weight markers.

### Specificity of Association

For Oak Ridge fish, differences in population genetic parameters corresponded to bioindicators of exposure and effect (body burdens or radionuclides, DNA damage, and embryo abnormalities) in the contaminated populations. In addition, the presence of the CIBs was associated with higher relative fecundity, fewer abnormal embryos, and less DNA damage in fish from radionuclide-contaminated populations. Although there was a tendency for these associations to be present for all CIBs, these patterns were not statistically significant in all cases. Furthermore, individuals with the NP allele 1 also had higher fecundity and fewer strand breaks than individuals that did not. The number of CIBs and heterozygous allozyme loci per fish were positively correlated with fecundity and negatively correlated with amount of DNA damage. Such associations were not seen for non-CIB loci or for fish from non-contaminated populations.

There were also environmental differences between sites that do not correspond to reference vs. contaminated streams; Pond 3513 is a small settling basin, Crystal Springs is a clear, cooler stream that originates from a groundwater spring and is impounded by a small weir, White Oak Lake is an impoundment of the warm-water White Oak Creek, and Wolf Creek is a warm-water creek that is more sluggish and turbid than Crystal Springs.

### Time Order or Temporality

The fact that Pond 3513 existed and was contaminated before it was colonized with mosquitofish is evidence for the time order criterion. Also, the use of two different nuclear DNA markers allows for comparison of population genetics using rapidly evolving and slowly evolving markers; allozyme markers are more evolutionarily constrained and are expected to evolve slower (because they are coding loci) than are RAPD markers. The fact that allozyme analysis indicates that Pond 3513 is more closely related to Crystal Springs, while RAPD markers indicate that Pond 3513 is more closely related to White Oak Lake, suggests that a recent evolutionary event has occurred that affected genetic relatedness of the populations.

### Biological Gradient

The level of radionuclides present in Pond 3513 is greater than that in White Oak Lake. Also, biomarkers (DNA damage and number of abnormal embryos) suggested that the level of contaminant effects in Crystal Springs was intermediate between White Oak Lake and Wolf Creek. The elevated biomarker responses in Crystal Springs could have been due to highway runoff (Crystal Springs was in a much more populated area than Wolf Creek) or to the fact that Crystal Springs originates from a subterranean cave, so the water that emerges from this cave is saturated with Radon gas (I.L. Larson, ORNL, pers. comm.). In any event, there is concordance in the trends of genetic diversity, frequency of CIBs and NP allele 1, average number of CIBs and heterozygous loci per fish, amount of DNA damage, and number of abnormal embryos. All of these endpoints decreased in the order of: Pond 3513/ White Oak Lake > Crystal Springs > Wolf Creek.

**Experimental Evidence**

The experimental evidence consisted of exposing mosquitofish to X-rays in the laboratory or collecting fish from Crystal Springs and caging them in Pond 3513. In both cases, the same association was found between the frequencies of CIBs and the level of DNA strand breakage. In addition, there was an association between survival and presence of CIBs in the fish caged in Pond 3513. In these experiments fish collected from Crystal Springs were caged in Pond 3513 or a noncontaminated for 6 weeks. There was a much higher mortality for Crystal Springs fish caged in Pond 3513 compared to those caged in noncontaminated ponds. There were no associations between survival or DNA damage with presence of CIBs in the nonexposed fish. These findings are consistent with the hypothesis that differences between Pond 3513 and Crystal Springs populations were due to selection for resistant genotypes. Again, there was a tendency for concordance between survival, DNA damage, and band presence for all CIBs, although these patterns were not always statistically significant. Also, the genetic distances between the resident Crystal Springs and Pond 3513 populations and the fish caged in Pond 3513 or the noncontaminated pond were determined before and after the exposures. Before exposure, all the caged fish were most genetically similar to Crystal Springs (the source of the fish for this experiment). After the exposure, the survivors caged in the uncontaminated pond were still most genetically similar to Crystal Springs, but the survivors caged in Pond 3513 were most genetically similar (on average) to the resident Pond 3513 population.

**Biological Plausibility**

There were several findings that indicated that the level of contamination in Pond 3513 was great enough to affect survival and reproductive success of the fish in fish introduced into this pond. First of all, the number of developmental abnormalities in embryos from the contaminated sites (Pond 3513 and White Oak Lake) was greater than that for the reference sites. The average fecundity was also lower in the contaminated sites, at least in the spring sampling period (Theodorakis *et al.* 1996). There was not only reduced survival of the fish caged in the contaminated sites, but also all of the fish caged in the noncontaminated site were gravid, while none of the fish caged in the noncontaminated site were gravid (unpublished data). Finally, it was noted that, upon introduction of the fish from Crystal Springs into Pond 3513, there was evidence of stress and high mortality (G. Blaylock, ORNL, pers. comm.).

The mechanism of radioresistance or association between genotype and fitness parameters (fecundity, survival) or DNA damage is currently unknown, as is the mechanism behind increased genetic variation in contaminated populations. However, there are four possible scenarios: (1) It is possible that the RAPD loci and the nucleoside phosphorylase locus are linked to genes that are involved in the radiation response. (2) Nucleoside phosphorylase activity is induced by radiation exposure (Hosek *et al.* 1991), suggesting a mechanistic link between expression at the nucleoside phosphorylase locus (a gene involved in nucleoside synthesis and DNA repair) and amount of DNA strand breaks. (3) The RAPD loci could be related to genotoxic response. Several studies have found that RAPD (or the related AP-PCR)

fingerprinting patterns can be altered by carcinogen-induced genomic instability (Keshava *et al.* 1999; Navarro and Jorcano 1999), heritable mutations (Kubota *et al.* 1992), or DNA damage (Atienzar *et al.* 1998, 1999, 2000; Conte *et al.* 1998; Savva 1998; Becerril *et al.* 1999). These findings suggest that at least some RAPD loci may be amplified from mutation/DNA damage hotspots, regions involved in genomic instability, or mobile genetic elements. RAPDs are also amplified from inverted repeats, and examples of inverted repeat elements associated with response or resistance to radiation exposure include transposable elements (Staleva Staleva and Venkov 2001) and nuclear matrix attachment regions (DNA sequences that bind to the nuclear matrix [nuclear cytoskeleton]; Schwartz and Vaughan 1993). (4) An increased level of genetic diversity may be adaptive when organisms are exposed to radiation or other pollutant stress. The findings of a positive correlation between allozyme heterozygosity and fitness, and a negative correlation between heterozygosity and amount of DNA strand breaks, are consistent with this hypothesis. Similar findings have been found by Kopp *et al.* (1992), in which fish with higher average heterozygosity were more resistant to acid stress.

The CIBs identified in the above studies have been cloned and used as probes in Southern blots of RAPD amplification products from various species, including humans, gulls, sea urchins. The *G. affinis* RAPD probes hybridize to RAPD bands in the other species that are of similar size, indicating that these bands are conserved in sequence and in size, suggesting a functional significance for these bands. However, DNA sequencing has not shed any light as to what the functional significance might be.

## CONCLUSIONS

Population genetic responses to environmental contamination are fully amenable to the seven causality criteria outlined in Adams (2003). There are a myriad of environmental factors other than environmental contamination that can affect population genetic structure, including environmental gradients, stochastic environmental variation, evolutionary history, barriers to/corridors for gene flow, habitat alteration. Consequently, establishing causality between contaminant exposure and population genetic effects requires a weight of evidence approach, employing multiple studies at various levels of biological organization (population genetics, environmental chemistry, organismal-level bioindicators or fitness parameters, and physiological, biochemical, and/or molecular biomarkers). In order to further illustrate this point, Table 1 summarizes how the studies of Theodorakis and coworkers can be applied to these causality criteria.

It is naive to believe that one can argue the case for pollution effects of population genetics based on results of the individual studies, or argue against such effects by isolating each study and pointing out weaknesses on a case-by-case basis. This is because each study by itself will probably be inconclusive and even the best will have weaknesses. Arguments based on such an approach are specious, whether they be used to refute (Belfiore and Anderson 2001) or support such population genetic effects, and are often motivated by preconceptions that the effects either do or do not occur. Consequently, each individual study is interpreted to support the preconception, but the prudent investigator avoids such a fallacy. A more rigorous ap-

**Table 1. Summary of the studies by Theodorakis and coworkers[a] as they apply to supplying evidence to support to the seven causality criteria [b] of pollutant cause and effects.**

| Causal Criterion | Supporting Evidence |
|---|---|
| Strength of Association | Multiple contaminated and reference sites<br>Lack of correspondence with evolutionary theory (Crystal Springs vs. Pond 3513) |
| Consistency of association | Parallel responses and homology between *Gambusia affinins* and *G. holbooki*<br>Parallel response among many RAPD bands<br>Similar findings (heterozygosity) to Kopp *et al.* (1992) |
| Specificity of association | Correspondence with bioindicators of exposure and effect<br>Genotype-dependant fecundity, abnormal embryos, and strand breakage.<br>Confounding factors do not covary with effects |
| Time order or temporality | Pond 3513 contaminated before introduction of fish<br>Genetic distance: RAPD vs. allozyme |
| Biological gradient | Trends in genetic diversity, frequency of CIBs and NP1 allele, DNA damage, embryo abnormalities, and level of contamination are concordant |
| Experimental evidence | Genotype-dependant survival (caging study) and DNA damage (caging and X-ray studies) |
| Biological plausibility | Contamination great enough to affect fitness<br>Homology between disparate species |

[a]Theodorakis *et al.* (1998, 1999), Theodorakis and Shugart (1997, 1998b).
[b]Adams (2003)

proach to assessing population genetic effects would be to consider all the studies as a whole, and, based on the preponderance of evidence, determine if there is a reasonable degree of certainty that effects do occur. In the end, this degree of certainty must be determined by the professional judgment of experienced investigators with expertise in both eco- and evolutionary toxicology.

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