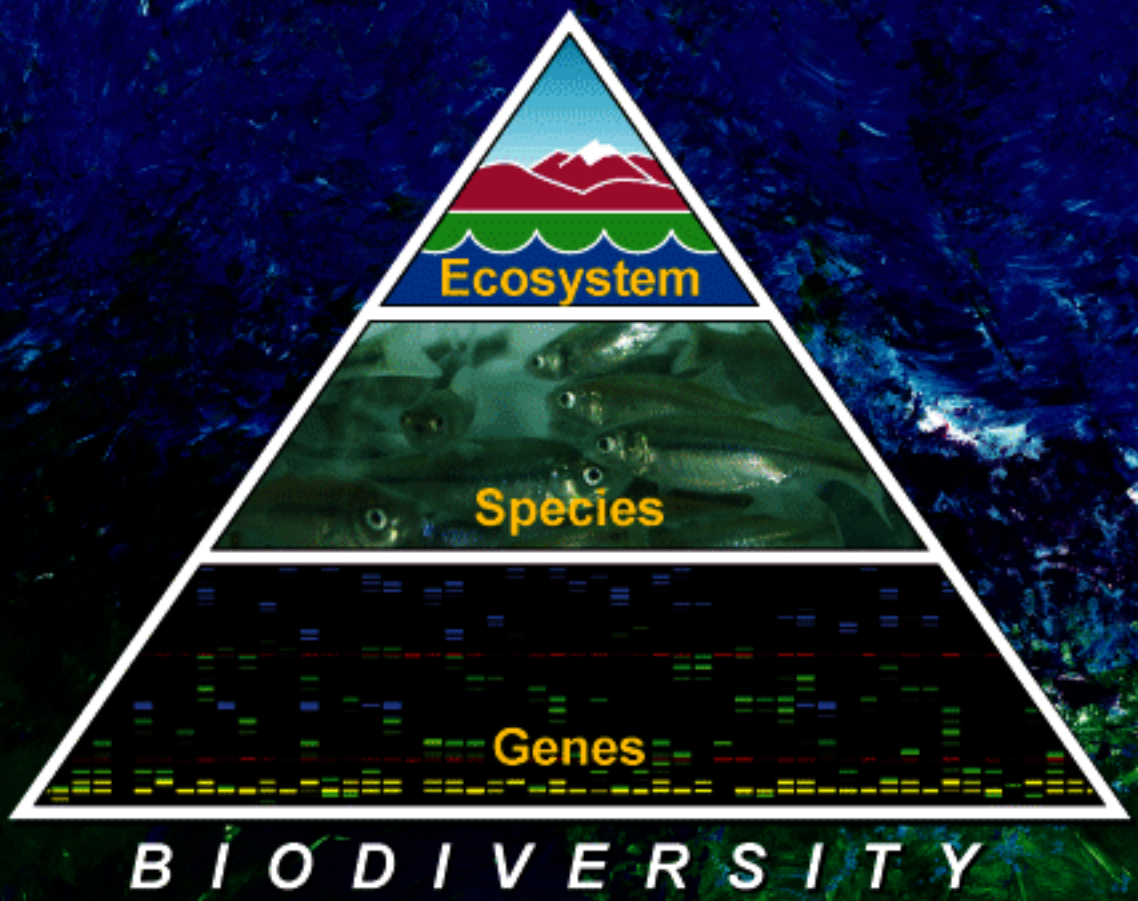
United States Environmental Protection Agency

Genetic Diversity as an Indicator of Ecosystem Condition and Sustainability



Utility for Regional Assessments of Stream Condition In the Eastern United States

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Genetic Diversity as an Indicator of Ecosystem Condition and Sustainability

Utility for Regional Assessments of Stream Condition in the Eastern United States

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Preface

In 1995, the U.S. Environmental Protection Agency's National Exposure Research Laboratory (NERL) enhanced its ecological research and development efforts to support the delivery of the "next generation" of biological indicators. These new ecological condition and stressor diagnostic indicators would follow the risk paradigm organization of the U.S. EPA's Office of Research and Development (ORD). ORD's Ecological Research Strategy (US EPA, 1998) called for the "development of a set of indicators for estuarine, stream, and lake systems that can be interpreted relative to status and changes in fundamental ecological and hydrologic processes that influence and constrain the integrity and sustainability of these systems." Genetic diversity indicator research was initiated in NERL to address this objective.

In 1998, ORD's National Center for Environmental Research (NCER) requested proposals from the academic research community for Science to Achieve Results (STAR) grants focused on "Ecological Indicators". Research sponsored by these grants emphasized genetic diversity and landscape ecology, both of which can be interpreted at a number of geographic scales, a requirement for the next generation of ecological indicators. In May 2000, NCER sponsored a review of genetic diversity science including both ORD scientists and STAR grant recipients during which a roundtable discussion delineated contributions as unique to either academic or federal laboratories. Large, regional-scale evaluations of genetic diversity within species, deemed beyond the scope of academic laboratories, were seen to be appropriate for federal laboratories and easily incorporated into existing environmental monitoring studies such as the Environmental Monitoring and Assessment Program (EMAP). These views were mirrored by recommendations in the July 1999 NSF Task Force on the Environment document "Environmental Science and Engineering for the 21st Century" which called for genetic diversity research within federal laboratories. Current advances in molecular biological science and technology have converged with classical genetic research, large-scale field biological monitoring, and remote sensing capabilities to provide unprecedented opportunities for multifaceted studies of species population structure and dynamics. These types of studies are critical to understanding the integrity and sustainability of ecosystems.

This report chronicles significant strides made in the development of an ecological indicator based on genetic diversity that is suitable for environmental monitoring studies at a range of geographic scales. Implementation of regional studies of genetic diversity required development of protocols for inclusion of genetic sampling in field studies and large-scale laboratory throughput of these samples. Robust statistical methods facilitated the meaningful interpretation of genetic diversity DNA fingerprinting data in the context of other environmental data collected concurrently. Documentation of these procedures for measuring genetic diversity is presented herein, along with the background and rationale for employing genetic diversity as an ecological indicator. Case studies are presented which demonstrate the application of genetic diversity in two field-monitoring efforts. Finally, recommendations are given for genetic diversity study design and technology transfer based on field and laboratory experience with large-scale studies.

Summary

Genetic diversity is a fundamental component of biodiversity and is as critical to sustainability of our natural resources as are diversity of species and ecosystems. It encompasses all of the genetically determined differences that occur between individuals of a species. Virtually all species are composed of populations that exist somewhat independently of each other, and thus genetic diversity exists both within and among populations. Levels of genetic diversity in any one population are determined primarily by four forces: (1) mutation, the ultimate source of all genetic diversity; (2) migration, the exchange of individuals between populations; (3) natural selection, the removal of "unfit" individuals from the population; and (4) genetic drift, random changes in gene frequency each generation due to limited numbers of breeding adults. The natural history of a species and the structure and dynamics of populations provide the arena in which these forces interact to drive evolutionary adaptation of populations to their environments. Thus, natural and anthropogenic environmental changes lead to changes in genetic diversity, both within and among populations, and genetic diversity measurement can provide insights into the consequences of environmental changes.

Genetic diversity can be measured by examining common morphological or morphometric traits. Such observable characteristics often result from the interaction of many genes, the expression of which is influenced by environmental factors. Assessments of molecular markers based directly on DNA have simple inheritance patterns and are not influenced by environmental factors. This simplification of the genetic system allows precise estimates of genetic diversity for any one marker and, by assessing many markers, can provide more precise estimates of overall levels of genetic diversity within and among populations of a species. Mathematical tools have been developed that allow diagnosis of the relative strengths of the four genetic forces and, indirectly, properties of populations, such as population size, breeding structure, and dispersal abilities.

Measurement of genetic diversity with molecular markers can add value to assessments of ecological condition derived from other ecological indicators, such as landscape and species assemblage indicators. Population parameters can be effectively estimated with molecular markers and used to characterize the geographic structure and connectivity of populations critical to interpreting data for ecological assessments. Genetic diversity also serves as an independent indicator of environmental condition. Environmental stressors typically reduce genetic diversity, primarily through the forces of selection and genetic drift, so that a recent reduction in genetic diversity is indicative of deteriorating environmental condition. As an indicator of ecological condition, genetic diversity integrates the genetic effects of multiple sources and is cumulative over time. In addition, it is a naturally 'scalable' indicator, as the geographic structuring of genetic diversity at the population, watershed, and regional levels is easily inferred.

The importance of genetic diversity to long-term sustainability is widely accepted, although the efficacy of molecular measures of genetic diversity for diagnosing extinction risk remains unclear and needs further investigation. Standing levels of genetic diversity in populations contribute to long-term sustainability in several ways. First, the ability of populations to adapt to changing environments is directly dependent on the amount of genetic diversity they possess. Second, small populations that lose genetic diversity may experience fitness reductions and increased extinction risk. Finally, populations that are adapted to

local conditions may become less fit if individuals from other areas that are adapted to different conditions are allowed to interbreed with them; the ensuing reduction in genetic diversity between populations influences long-term sustainability of the species.

This report documents research undertaken to determine if the theoretical promise of genetic diversity as an ecological indicator is realized in real-world applications. Results of two case studies confirm that genetic diversity is a useful indicator of environmental condition. The first case study incorporated the genetic diversity indicator in a larger Regional Environmental Monitoring and Assessment Program study of the Eastern Cornbelt Plains Ecoregion, done in collaboration with US EPA Region 5 and Ohio EPA. Genetic diversity of a small cyprinid minnow, the central stoneroller (Campostoma anomalum), was measured at 91 sites in nine watersheds using the RAPD (random amplified polymorphic DNA) fingerprinting technique. Although the RAPD technique was chosen primarily for ease of technology transfer, experiences with the technique suggested that it might not be robust to the normal variations in equipment and technical skills that exist among different laboratories. Nonetheless, the genetic diversity data obtained proved highly informative. Although sample sizes varied and were sometimes small (3-10 individuals per site), large differences in genetic diversity within sites and among sites were detected. Significant differences in the average levels of genetic diversity within populations were observed among major river drainage basins, leading to the conclusion that populations of stonerollers are highly differentiated within the Eastern Cornbelt Plains Ecoregion and there is geographical structuring of these populations within and among watersheds. Genetic diversity is related to environmental condition, particularly impacts from urbanization, channelization, and impaired riparian zones. Expected relationships between genetic diversity and existing ecological indicators such as the IBI and QHEI were seen, although the small degree of correlation suggests that the genetic diversity indicator provides supporting and not highly redundant information for environmental condition assessments.

The second case study examined the genetic diversity indicator applied to populations of the creek chub (Semotilus atromaculatus) in a small region of western Pennsylvania and West Virginia underlain by coal-bearing geology and for which the history of coal mining operations is known. Samples of between 9 and 28 creek chubs were collected from 10 sites within 4 watersheds. Two molecular methods were used: the amplified fragment length polymorphism (AFLP) fingerprinting technique was used to assess diversity in the nuclear genome, while a portion of the mitochondrial genome was assessed using DNA sequencing. Mitochondrial DNA differences showed a strong spatial component. The nuclear DNA also differentiated the populations although the genetic structure was not as strong as that seen in the mitochondrial DNA. Environmental factors (derived from principal components analysis of 25 key environmental measurements) accounted for about half of the differences in mitochondrial DNA diversity and virtually all of the differences in nuclear DNA (AFLP) diversity.

These two case studies clearly demonstrate that genetic diversity can serve as an indicator of environmental condition. They also provided the practical experience upon which recommendations for future implementation are based.

At present, genetic diversity indicators will be used most effectively if they are incorporated into multi-indicator assessments at large, regional scales. Typically, reduced genetic diversity in particular popula-

tions is inferred from assessments of spatially separated populations, although it can be detected more easily through temporal monitoring. Thus, incorporation of genetic diversity indicators into monitoring programs at intensively studied index sites will be useful. Regional genetic diversity data can then serve as baseline data for future monitoring of temporal patterns in genetic diversity at different spatial scales.

A number of different molecular technologies can be used for genetic diversity analysis, including allozyme, DNA fingerprint, microsatellite DNA, and mitochondrial DNA fragment or sequence analysis. At present, it appears that the most cost-effective strategy is to incorporate microsatellite markers into existing or planned ecological assessments. It may be beneficial to supplement microsatellite analysis with mitochondrial DNA analysis since mitochondrial DNA can yield complementary information. This approach is the most technologically challenging of all the genetic diversity assessment options. Thus, the recommendation is that a "three-laboratory approach" be used to obtain and interpret genetic diversity data. The lead lab would be the regional field lab, which would be responsible for design of the assessment, field collections, and preparation of DNA samples. A marker development laboratory would design molecular markers specific for the target species identified by the regional lab. A genetic analysis laboratory would use the molecular markers and DNA samples obtained from the other labs to perform the genetic diversity assessment and, together with the regional laboratory, derive the ecological assessment.

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1. Introduction

Historically, the U.S. Environmental Protection Agency and other governmental agencies charged with monitoring and safeguarding the quality of aquatic environments tended to concentrate their efforts on assessment of the immediate toxicological effects of discharges from known pollution sources. Gradually, as the more flagrant pollution sources were identified and removed, it became clear that much of the remaining pollution in our waterways could not be ascribed to specific point sources but was generated at low levels from many nondescript sources. In addition, there was concern that aquatic ecosystem health was not a simple function of water quality and could not be fully measured based on the results of a series of short, standardized laboratory tests. Following the publication of an influential report over a decade ago (U.S. Environmental Protection Agency, 1988), the EPA began to shift its ecological research to focus attention on ecosystem responses to cumulative, regional, and long-term anthropogenic disturbances. The EPA's Environmental Monitoring and Assessment Program (EMAP) was born of this movement, and was given the mission to evaluate the status and trends in the condition of the Nation's ecological resources (Messer, *et al.*, 1991).

A number of ecological indicators have since been developed that have, in one fashion or another, aided in measuring the overall health of aquatic ecosystems. These include indicators based on types of assemblages of species present and landscape or land cover indices. The application of these indicators has clearly expanded our toolbox for monitoring the status of aquatic ecosystems. Nonetheless, important information about the current status and predicted future status of the Nation's aquatic ecosystems still remain to be addressed. For example, current indicators tell us little about the biological independence or connectivity of different geographic areas. How important to ecosystem health are the natural migration barriers and corridors that separate and connect different populations? If populations of a species from one region are extirpated, will individuals that are similarly adapted to that environment replace them? How much of the evolutionary history of a species will be lost if the habitat of one distinct population is destroyed to make room for a new development?

In addition, we know little about the many hidden effects of anthropogenic stressors on species and ecosystems that do not manifest as gross deformities, dead or dying fish, or extinct species. Do these stressors evoke heritable changes in organisms that may affect the sustainability of populations many generations into the future? If past or current stressors have altered the genetic characteristics of populations, have these changes affected their ability to withstand the additional stresses of future human population growth, global warming, and associated environmental modifications? Are some populations of a species genetically "pre-programmed" to withstand severe environmental modifications while others are not?

These are important questions that must be answered to truly understand the condition and sustainability of the Nation's ecological resources. Clearly, they cannot be answered using simple streamside assays or laboratory tests. To answer these questions, we must come to understand the dynamics of populations and how those dynamics will be altered in the face of anticipated environmental changes. To understand the long-term effects of environmental changes on populations, we must learn how environmental modifications impact genetic diversity within and among populations and how changes in genetic diversity ultimately influence the ability of populations to withstand further environmental change.

1.1 What is Genetic Diversity?

The term 'genetic diversity' has been defined in various ways to suit different purposes. Here, we restrict our interests to intraspecific genetic diversity, which represents the range of heritable differences of a trait

or set of traits among individuals within a species and includes diversity among individuals within populations as well as variation among different populations (Figure 1-1). The term is essentially synonymous with 'genetic variation' or 'genetic variance'. As defined, we consider genetic diversity a trait of populations and species, not of individuals. Examples include variation in the genetic determinants of eye color, growth rate, and disease resistance within and among populations. Similar variation can be assessed in specific stretches of DNA using molecular markers. Genetic diversity is one of the three components of biodiversity, along with species diversity and ecosystem diversity. In a sense, it is the fundamental organizational component of biodiversity, since species diversity is built from genetic diversity, and ecosystem diversity derives from species diversity. Thus, any goal to monitor or maintain biodiversity is incomplete and superficial if it considers only species and ecosystems.

Genetic diversity is shaped by past population processes and affects the sustainability of species and populations in the future. Physical, chemical, and biological environments are constantly changing over short and long time scales. To keep up with changing environments, species must adapt or face extinction. The ability of species to adapt to altered environments is directly related to the amount of genetic diversity available to natural selection. For two populations that are under similar environmental pressures to adapt, the population with the greater genetic diversity for fitness traits is expected to adapt more quickly. That the maintenance of genetic diversity is key to the long-term survival of most species is a central paradigm of the field of conservation genetics (Soule, 1987).

The main forces that determine current levels of genetic diversity within species are: mutation, migration, selection, and genetic drift (Figure 1-2). Mutation is the ultimate source of all genetic diversity, but is usually a relatively weak force in comparison to the other three. Most new mutations are either neutral or harmful with respect to fitness. A very small proportion of mutations is expected to increase fitness.

Migration or gene flow represents the movement of breeding individuals between populations. It is usually a very strong force on genetic diversity, although it can be weak for species with low dispersal abilities. Disturbance of a species' migration regime may have substantial effects on fitness. In general, migration has the effect of increasing genetic diversity within populations and homogenizing genetic differences among populations.

Natural selection, along with genetic drift, is one of the main forces that cause separate populations to become differentiated. It is the primary force that increases the average fitness of populations. Selection can have variable effects on genetic diversity, depending on the form of selection that is operating. In cases where an environmental change has caused a different set of genes or genotypes to be optimal, selection is expected to decrease genetic diversity at selected loci, at least until the population mean has shifted to the new genetic optimum.

Genetic drift represents the random change in gene frequencies in each generation due to a finite number of breeders producing each new generation. It is the statistical equivalent of sampling error. Without the input of new mutations, all genetic diversity within species would eventually be lost due to the effects of genetic drift. Genetic drift tends to be a very strong force on genetic diversity in small populations, even for loci that are under intense selection. As a consequence, adaptation is more difficult in small populations while random fixation of deleterious alleles that would normally be removed by selection is more likely. The effect of genetic drift in large populations is weaker but may still be a dominant force for loci that are not under selection. Genetic drift has the effect of decreasing genetic diversity within populations and increasing genetic diversity among populations.

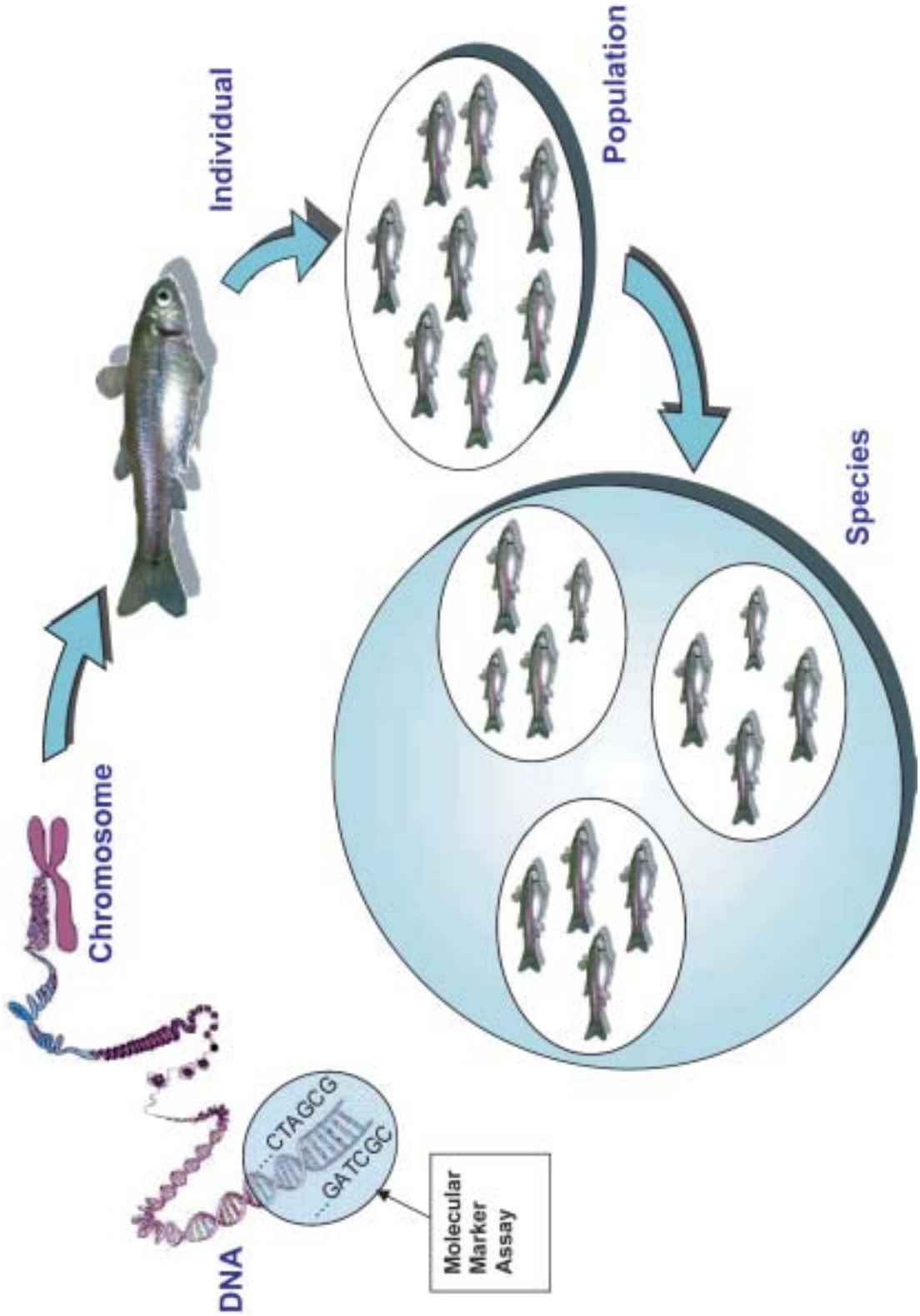

Figure 1-1. Genetic diversity arises from mutations and recombination at the molecular DNA level or from chromosome rearrangements at the cytological level. Molecular markers assay this DNA variability. The sum-total of all the genetic material determines the genotype, the genetic contribution to the individual's phenotype (physiological, morphological, and behavioral attributes). Genetic diversity within populations represents the variation in the genotypes of different individuals in the population. Genetic diversity within the species also is affected by differences in genotype frequencies among the populations.

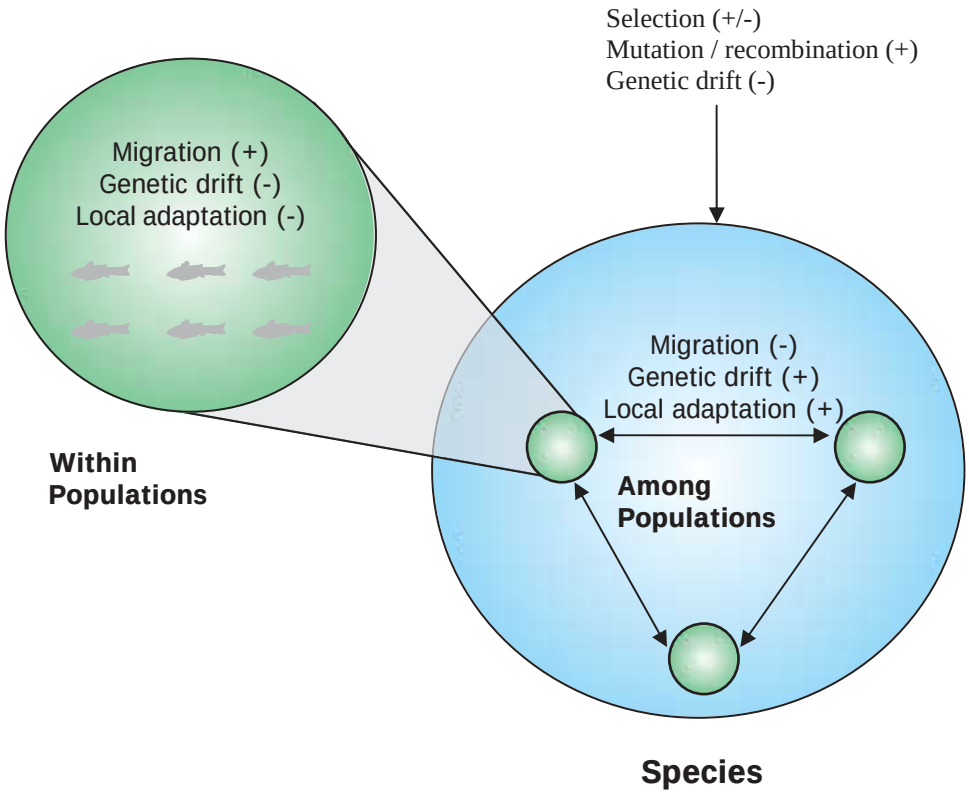

Figure 1-2. Forces that influence overall (species-level) genetic diversity, as well as components of genetic diversity among and within populations. Forces that are annotated with a + generally increase genetic diversity; those annotated with a - generally decrease diversity. Selection may either increase or decrease genetic diversity, depending on specific circumstances.

2 Rationale for a Genetic Diversity Indicator

It is now a widely accepted practice to integrate genetic management ideas into resource management planning (e.g, Hessel, 1992; Moritz, 1994). The primary goal of genetic management is to ensure that sufficient genetic diversity is retained in order to maintain short-term fitness and long-term sustainability and to ensure that local adaptations are not lost due to intermixing of previously distinct populations. An additional goal is to identify and protect those populations that represent highly distinct or important evolutionary lineages, because if such populations become extinct, an important component of the genetic diversity of the species would be lost. The idea that genetic monitoring can be used as an indicator of environmental condition is less prevalent in resource management plans (salmon management in the Pacific Northwest being a notable exception), primarily because most resource agencies still consider genetic techniques to be novel, expensive, or technically challenging. This has created the somewhat paradoxical situation in which resource managers pay close heed to conservation genetic principles, but have not

adopted policies to measure the success of management actions in maintaining genetic diversity. Nonetheless, an enormous database of genetic diversity studies exists in the scientific literature and can provide guidance in the development and evaluation of genetic diversity indicators.

One of the most important uses of a genetic diversity indicator for environmental assessments is simply to characterize the natural genetic structure of component populations within ecosystems.

2.1 Relevance of genetic diversity to assessment of ecological condition

The genetic diversity of populations responds to environmental heterogeneity via alterations in the relative strengths of the four opposing genetic forces: mutation, migration, selection, and genetic drift. The balance and cumulative history of these forces determines actual levels of genetic diversity at any one time. The accuracy and sensitivity of measurements of genetic diversity has steadily increased with advances in molecular marker technologies. An enhanced ability to describe patterns of genetic diversity in space and time has, in turn, made it easier to diagnose the genetic mechanisms that produce these patterns (Carvalho, 1998).

By far, the easiest genetic forces to measure in natural populations are equilibrium levels of migration (gene flow) and genetic drift. Gene flow provides a direct measure of the evolutionary connectivity of populations while genetic drift provides a measure of effective population size. Populations that have low connectivity with others become genetically differentiated and more unique. These populations are often assigned higher conservation 'value' because a large portion of the genetic diversity of the species would be lost if they were extirpated (Moritz, 1995). Populations with low effective population size may be more vulnerable (see section 2.2) and therefore may be targeted for immediate conservation efforts. The use of genetic markers to measure connectivity and effective population size and to assign conservation values and priorities is commonplace, as evidenced by numerous articles in scientific journals such as *Molecular Ecology, Evolution, Conservation Biology,* and *Conservation Genetics*. Thus, the concept that genetic diversity is a useful indicator of population structure and conservation status is well established. However, in these studies, evaluations of genetic diversity are almost always focused narrowly on the status of particular populations residing in specific habitats; inferences to overall ecological condition based on genetic diversity are not usually made.

For the purpose of performing environmental assessments, one of the most important potential uses of a genetic diversity indicator is simply to characterize the natural population structure of component populations within ecosystems. The effective population size and connectivity of populations are fundamental attributes of populations that can be diagnosed only with genetic markers. Because populations are the basic biological units that respond to changes in the environment, delimitation of population boundaries is critical to effective ecosystem monitoring and management.

Without this basic knowledge of population structure, it is impossible to judge the extent or range over which local or regional environmental impacts will affect ecosystems. In addition, concordance of population genetic structure across several species may indicate biogeographic boundaries not recognized through analysis of species distributions alone (Moritz and Faith, 1998). By defining population boundaries, genetic diversity indicators provide fundamental data that enhance the value and interpretation of other ecological assessment data, such as those obtained from landscape and species assemblage indica-

tors.

In addition to delineating these long-term, evolutionary characteristics of populations, a useful indicator of ecological condition should be responsive to environmental change (US EPA, 1998). Genetic diversity responds to environmental change, primarily through the actions of genetic drift and selection, but also through mutation and gene flow. The indicator 'remembers' population effects. In other words, once genetic diversity is reduced it will remain low until mutation or gene flow replenishes it (Figure 2-1). Because the effects of selection and drift are cumulative across generations, there is often a lag time before significant changes in genetic diversity are revealed (Bickham *et al.*, 2000). Thus, a genetic diversity indicator is expected to be useful primarily for multigenerational exposures; the relevant timescale for indicator response is years or decades, not months. Exceptions occur when populations are severely bottlenecked or severe selection pressures are imposed on populations, inducing rapid and severe changes in genetic diversity. Decreases in genetic diversity in response to experimental bottlenecks of laboratory populations are well known (Hartl and Clark, 1997). In general, these studies show that declines in heterozygosity at marker loci follow theoretical predictions regarding the loss of genetic diversity in relation to effective population size. "Natural" population bottleneck experiments, such as those that occurred when elephant seals were hunted to near extinction and when European starlings were introduced to North America, also show a remarkable correspondence with genetic theory (Cabe, 1998; Hoelzel, 1999).

Genetic diversity indicators provide fundamental data that enhance the value and interpretation of other ecological assessment data, such as those obtained from landscape and species assemblage indicators.

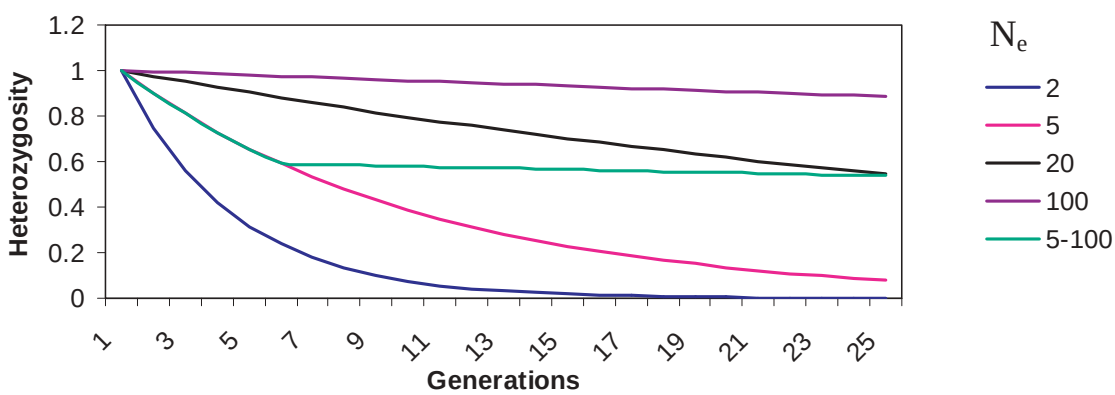

Figure 2-1. Predicted loss of heterozygosity over time from genetic drift. Heterozygosity (a measure of genetic diversity within populations) decreases more rapidly in populations with smaller effective population sizes (N_e). For the series labeled "5-100", the population started at an effective size of N_e=5 and remained there for five generations. Starting at generation 6 the effective size grew to N_e=100. Note that information about the past bottleneck continues to be reflected by heterozygosity after the population has rebounded.

Numerous field studies have demonstrated a correlative relationship between levels of genetic diversity and single or mixed contaminant exposures. Examples of changes in the genetic structure of plant populations exposed to mine wastes are well known, and were recently reviewed by Shaw (1998). Several examples of correlations between genetic diversity and contaminant exposures for freshwater and marine species are listed in Table 2-1. In most of these cases, the frequency of a particular allele or genotype shift-

ed dramatically in exposed populations relative to frequencies in reference populations. Often, a similar shift was observed for the same marker locus (e.g., the allozyme locus GPI-2) in different species, suggesting that selection was operating at that particular marker (Gillespie and Guttman, 1999). In addition, genetic diversity of exposed populations was often lower than for reference populations, suggesting that the effective sizes of exposed populations were reduced thereby inducing strong genetic drift effects. In at least one case, a significant difference in genetic diversity between reference and exposed populations was observed when indicators based on species assemblages suggested homogeneity (Roark and Brown, 1996).

The scientific literature … shows a clear relationship between genetic diversity and ecological stressors that is consistent with genetic theory.

Many factors can contribute to differences in allele frequencies so demonstration of population differences does not, by itself, convincingly demonstrate that the specific contaminants directly impacted genetic diversity. However, a causal link between susceptibility and resistance to specific contaminants and genetic diversity has been demonstrated in several laboratory and mesocosm experiments (Table 2-1). For example, Gillespie and Guttman (1989) found that central stonerollers with allozyme genotypes that were common in contaminated environments had greater survival following laboratory exposure to copper than those with other genotypes. While significant effects on genetic diversity have been observed for a large number of organisms and experimental exposures, other studies have failed to identify significant genetic changes, even in cases where correlations were observed in natural populations (e.g., Diamond *et al.*, 1989). Although a number of potential reasons can explain the lack of genetic response in these studies, they demonstrate that genetic diversity is as yet an imperfect indicator of exposure.

Besides chemical inputs, other anthropogenic stressors that affect survival or selection would be expected to impact genetic diversity as well. In practice, few examples of genetic diversity assessments relative to recent environmental alterations have been reported. An exception is the rather large number of studies that have documented the strong effects on genetic diversity of interspecific and intraspecific hybridization due to species introductions (e.g., Campton and Johnston, 1985; Gyllensten *et al.*, 1985; Leary *et al.*, 1995; Williams *et al.*, 1996; Ayres *et al.*, 1999). The effect of hybridization on genetic diversity is immediate and, if the hybridizing taxa are genetically distinct, easy to detect with genetic markers. The effect of habitat fragmentation on genetic diversity also has been investigated. Increased genetic differentiation of populations in relation to habitat shrinkage has been reported for woodpeckers (Haig *et al.*, 1996), lizards (Schneider *et al*, 1998), mint plants (Morden and Loeffler, 1999), and beetles (Knutsen *et al.*, 2000). The construction of a highway was reported to have increased genetic differentiation between vole populations on each side of the road (Gerlach and Musolf, 2000). Smith *et al.* (1983) reported results of a survey of genetic diversity in mosquitofish that suggested genetic differentiation due to water impoundments. If population size within fragments is decreased, fragmentation should result in lower genetic diversity within populations as well, but this was either not observed or not reported in these studies.

Although there is much interest in measuring the ecological effects of environmental mutagens and carcinogens, studies of the heritable effects of these exposures on natural populations are extremely rare. However, measures of genomic mutation rates and inferences to possible changes in mutation rates, both globally and in specific environments, are extremely important. Population genetics theory suggests that even a very small increase in the mutation rate may decrease the fitness of many populations enough to

Table 2-1. Natural populations and laboratory or mesocosm studies of freshwater and marine taxa that indicate a correlation between genetic diversity and exposure to contaminants (modified and extended from Gillespie and Guttman, 1999).

Known Stressors	Molecular Marker	Taxa	Natural Population (NP) Or Laboratory / Mesocosm (L/M) Study	References
Mercury, other metals	Allozyme	Gastropods	NP	Nevo *et al.*, 1984
			NP	Benton *et al.*, 1994
		Shrimp	NP	Nevo *et al.*, 1984
		Fish	NP	Heagler *et al.*, 1993
			NP	Keklak *et al.*, 1994
			NP	Benton *et al.*, 1994
			NP	Roark and Brown, 1996
		Fish	L/M	Diamond *et al.*, 1989
			L/M	Heagler *et al.*, 1993
			L/M	Mulvey *et al.*, 1995
			L/M	Tatara *et al.*, 1999
		Shrimp	L/M	Ben-Shlomo and Nevo, 1988
		Gastropods	L/M	Lavie and Nevo, 1986b
		Insect	L/M	Benton and Guttman, 1992a,b
		Fish	L/M	Chagnon and Guttman, 1989
			L/M	Schlueter *et al.*, 1995, 1997, 2000
		Amphipod	L/M	Duan *et al.*, 2000a
		Shrimp	L/M	Ben-Shlomo and Nevo, 1988
		Gastropod	L/M	Lavie and Nevo, 1982, 1986a,b
		Mollusc	NP	Moraga *et al.*, 2002
		Fish	NP	Larno *et al.*, 2001
Acidity, [Al]	Allozyme	Fish	NP	Kopp *et al.*, 1992
		Fish	L/M	Kopp *et al.*, 1992
		Amphipod	L/M	Duan *et al.*, 2000a
Arsenate	Allozyme	Fish	L/M	Newman *et al.*, 1989
Pesticides	Allozyme	Fish	NP	Hughes *et al.*, 1991
		Fish	L/M	Brown Sullivan and Lydy, 1999
		Bivalve	L/M	Tanguy *et al.*, 1999
PAH (fluoranthene)	Allozyme	Fish	L/M	Schlueter, *et al.*, 2000
		Amphipod	L/M	Duan *et al.*, 2000b
	Allozyme	Fish	NP	Larno *et al.*, 2001
Radionuclides	RAPD, Allozyme	Fish	NP	Theodorakis and Shugart, 1997
	RAPD	Fish	L/M	Theodorakis and Shugart, 1998
Overall water quality, complex effluents	Allozyme	Fish	NP	Gillespie and Guttman, 1989, 1993
			NP	Foré *et al.*, 1995a,b
			NP	Heithaus and Laushman, 1997
	CYP1A Sequence	Fish	NP	Roy *et al.*, 1996
	MtDNA/RAPD	Fish	NP	Murdoch and Hebert, 1994
	MtDNA	Copepod	NP	Street and Montagna, 1996
	RAPD	Fish	NP	Nadig *et al.*, 1998
		Crayfish	NP	Krane *et al.*, 1999
	RAPD	Mussel/Barnacle	NP	Ma *et al.*, 2000
	Allozyme	Fish	NP	Roark *et al.*, 2001.

drive them to extinction (Lynch, 1996). Measurement of mutations with highly mutable molecular markers (e.g., microsatellites, minisatellites) can reveal differences in mutation rates at both the individual and population levels. At the population level, an increase in the mutation rate is expected to result in a large frequency of very rare alleles. In practice, it may be more straightforward to measure the accumulation of new mutations directly, by assessing genotypes of parents and progeny, or by assessing the frequency of novel genotypes in gametes of reproductive individuals. For example, analysis of multi-locus DNA fingerprints of herring gulls and their offspring from an industrialized urban harbor indicated higher mutation rates than for gulls originating from areas with lower anthropogenic inputs (Yauk and Quinn, 1996). A similar analysis of microsatellite loci in barn swallows near Chernobyl, demonstrated two-to-tenfold higher mutation rates near the radioactively contaminated area (Ellegren *et al.*, 1997).

The scientific literature reviewed in this section shows a clear relationship between genetic diversity and ecological stressors that is consistent with genetic theory. In general, different stressors should have predictable consequences for measurements of genetic diversity. This is not to say that genetic diversity is likely to be useful to diagnose specific stressors, but that an established cause and effect relationship exists. Thus, loss of habitat that causes a decrease in the rate of migration between populations (fragmentation) is expected to increase the component of genetic diversity among populations, which may be measured as a decrease in the average similarity between populations or an increase in estimates of one of Wright's (1978) F-statistics, FST (Figure 2-2). If fragmentation results in small breeding population sizes, then a second predicted outcome would be reduced within-population genetic diversity or heterozygosity (H), and reduced numbers of segregating alleles per locus (N_a). Similarly, significant degradation of habitat due to contaminant exposure, siltation, changes in flow regime, or other modifiers that greatly reduce population size are expected to decrease genetic diversity within populations (reduced H, N_a) and greatly reduce genetic diversity of genetic markers that are linked to loci affecting adaptation to the altered habitat. The predicted effect of introduced species depends on whether they can successfully hybridize with native populations. Thus, introduced species are expected to have an effect similar to habitat degradation (decreased H, N_a) due to competition or predation but will increase genetic diversity through hybridization. Exposure to environmental mutagens is predicted to increase the average number of alleles at a marker locus (N_a) but, since each new mutation will be rare in the population, should have little effect on heterozygosity.

One of the principle advantages of genetic diversity measures is that they are highly scalable indices. For aquatic populations, the basic units of measure are populations that reside in different stretches of streams or rivers that are nested within watersheds that are nested within larger regions. Measures of genetic diversity, such as Wright's F statistics or Nei's genetic diversity statistics (reviewed in Nei, 1987) are especially appropriate for analyzing this hierarchical structure. For example, these population statistics can be used to assess the proportion of all genetic diversity that resides within populations, within local watersheds, within larger river drainages, and within regions. In fact, the only limitation to the number of hierarchical levels of analysis that can be assessed are biological, as the range a species can occupy ultimately limits the regional application of genetic diversity indicators.

Another important advantage of a genetic diversity indicator is that it naturally lends itself to analysis of temporal trends. In fact, temporal monitoring of genetic diversity, either regionally or at index sites, is the preferred application of the indicator (see section 3.1.1). Temporal monitoring provides the most direct and most effective measure of actual changes in the level or structure of genetic diversity. Analytical techniques have been developed to estimate the effective number of breeding adults from temporal changes in genetic diversity (Waples, 1990; Jorde and Ryman, 1996).

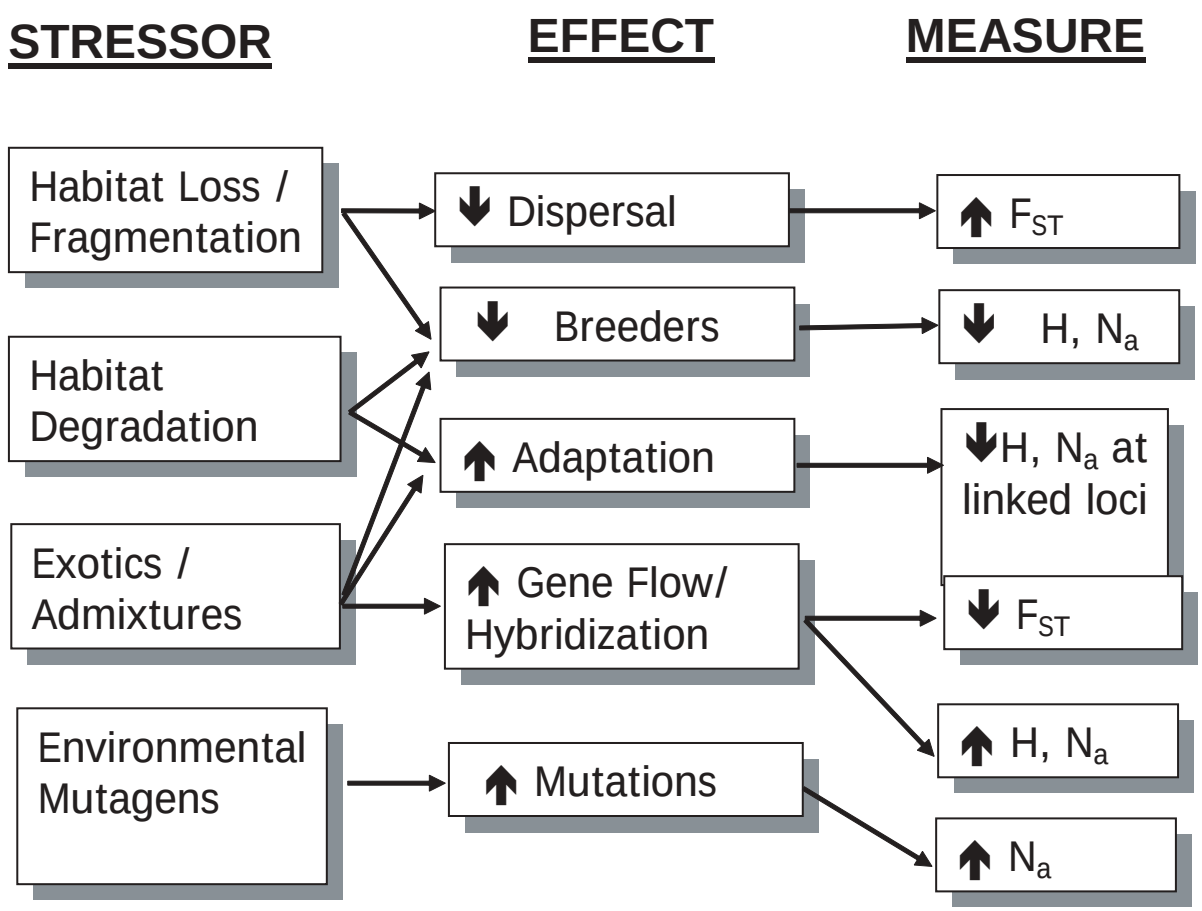

Figure 2-2. Population-level effects of different stressors and their predicted effects on measurements of genetic diversity. FST is a measure of population subdivision. Heterozygosity (H) and the number of segregating alleles (N_a) are measures of genetic diversity within populations.

Some characteristics of genetic diversity measures that support its use as an indicator of ecological condition are summarized in Table 2-2.

Table 2-2. Rationale for indicator of ecological condition based on genetic diversity

- Population-level measure rather than individual-level measure
- Measure of cumulative impact of multiple stressors on populations
- Integrative measure at multiple geographic and temporal scales
- Can be implemented as a nondestructive measure (e.g., DNA can be taken from fin tissue or scales)
- May identify problems within species before species assemblage indicators become significant
- Well-defined relationships between indicator and the size and connectivity of populations
- Highly complementary to species assemblage and landscape indicators
- Useful indicator of population trends through temporal monitoring
- Quantitative measure of population and community "conservation worth"

2.2 Relevance of genetic diversity to assessment of population sustainability

Genetic diversity is both an input and an output of ecosystems. As shown in the previous section, its role as an output tells us something about the processes occurring within ecosystems. As an input, genetic diversity tells us something about the future sustainability of ecosystems. The importance of genetic diversity for sustainability of ecosystems is due to its role in determining population fitness in the short term and population sustainability in the long term.

Due to rapidly increasing human pressures, current rates of environmental modification may be greater than those most modern species have experienced. For populations with little genetic variation, we expect that the rate of adaptation to altered environments will be slow. If the rate of environmental change is too great, then limitations on genetic diversity combined with high demographic costs of selection will not allow populations to replace their numbers each generation and the populations will slowly slide towards extinction (Lande and Shannon, 1996; Lynch, 1996; Lande, 1999). Rapid anthropogenic changes such as global warming are expected to greatly increase the importance of genetic diversity to population persistence in the foreseeable future (Lande, 1999).

Genetic Diversity is informative about ongoing processes occurring within ecosystems and about the future sustainability of those ecosystems

Besides loss of adaptive potential, low genetic diversity within populations can have an immediate effect on overall fitness. Experimental evidence suggests that most, if not all, diploid species harbor recessive lethal alleles and partially recessive sublethal alleles (Lande, 1999). If genetic diversity is reduced rapidly, strong genetic drift will result and many individuals will be homozygous for the (sub)lethal alleles, allowing them to be fully expressed. If the population size becomes very small, sublethal alleles may actually become fixed (all individuals homozygous for the deleterious allele), causing a permanent decrease in fitness and, consequently, increasing extinction risk. The effect is often called "inbreeding depression" since it is associated with inbred populations in laboratory experiments. The effects of inbreeding depression on population survival can be severe; most experimental populations of animals that are purposefully inbred will eventually become extinct due to continuous erosion of fitness (Soule, 1980; Frankham, 1995). On the other hand, a more gradual decline in genetic diversity may allow (sub)lethal alleles to be purged by selection without greatly harming fitness.

A correspondence between loss of genetic diversity at marker loci and reduced fitness has been difficult to document in wild populations, primarily because it is difficult to quantify population fitness in natural populations. Nonetheless, evidence for a relationship between particular fitness components and heterozygosity has been shown in a number of species (reviewed in Allendorf and Leary, 1986). More recent studies also suggest that a relationship between genetic diversity and fitness holds for a number of natural populations (Table 2-3). In only one case, however, was a direct relationship between genetic diversity and population extinction observed.

In addition to genetic diversity within populations, maintenance of genetic diversity among populations is important to the long-term sustainability of many species. Populations of many species are adapted to unique local conditions. In the long run, continued evolution of the species depends on these unique populations and a major conservation effort is dedicated to seeking out and protecting these 'evolutionarily significant' lineages. Removal of barriers to dispersal, either through habitat modification or translocation of

Table 2-3. Examples of genetic diversity studies of natural populations that demonstrated an association between genetic diversity and population fitness.

Endpoint	Molecular marker	Taxon	Reference
Local population extinction	Allozyme, microsatellite	Butterfly	Saccheri *et al.*, 1998
Lifetime breeding success	Microsatellite	Red deer	Slate *et al.*, 2000
Colony growth and survival	Allozyme	Ant	Cole and Wiernsasz, 1999
Fertility, hatching rate	Microsatellite	Prarie chicken	Westemeir *et al.*, 1998; Bouzat *et al.*, 1998
Mortality, growth, fecundity, developmental stability	Allozyme	Topminnow	Quattro and Vrijenhoek, 1989
Developmental stability	mtDNA, microsatellites	Elephant Seal	Hoelzel *et al.*, 2002
Male reproductive success	Allozyme	Butterflies (2 species)	Carter and Watt, 1988
Male reproductive success	Allozyme	Gastropod	Rolan-Alvarez *et al.*, 1995
Birth weight, neonatal survival	Microsatellite	Seal	Coltman *et al.*, 1998

individuals, allows the transfer of locally maladaptive genes into populations, thereby reducing population fitness. This effect is sometimes called "outbreeding depression". It is believed that the widespread practice of rearing fish stocks to supplement wild populations may have inadvertently reduced fitness and caused local extinctions of many fish populations (Nehlsen *et al.*, 1991). In addition, previously isolated but reproductively compatible species have been allowed to come into contact, creating "hybrid swarms" (Leary *et al.*, 1995). In the worst cases, native species diversity may be overrun by genes from hybridizing introduced species, effectively eliminating native species through dilution.

Long-term sustainability is affected by the ability of populations to adapt to changing environments. Gilpin and Soule (1986) summarized the theoretical relationship between genetic diversity, adaptation, demography, and population extinction in the "extinction vortex" model. Under normal circumstances, a temporary decrease in population fitness due to a change in the environment is corrected via the effects of selection acting on genetic diversity. The population is able to "track" the environment as it changes. However, under circumstances of low genetic diversity where insufficient alleles are available for selection, adaptation will be slowed and inefficient (Figure 2-3). Instead, the population size will be reduced due to poor fitness, which reduces genetic diversity and, in turn, reduces fitness. The separate processes of fitness loss, population loss, and demographic effects feed off each other until the population finally crashes.

While the concept that adaptation is dependent on genetic diversity is a cornerstone of evolutionary biology, there is little empirical evidence that measurements of genetic diversity are correlated with long-term sustainability or adaptive potential. Difficulty in defining and measuring adaptive potential may partly explain the present lack of evidence. However, a more fundamental problem regarding inferences made from measurements of genetic diversity with molecular markers also should be considered. Most molecular markers are selected and analyzed because they show sufficient variation to elucidate patterns of genetic variation within and among populations. With a few exceptions (e.g., some cases in Table 2-1), little or nothing is known about the direct influence of these molecular markers on fitness, although the

vast majority is believed to be effectively neutral (unselected). Neutral markers are preferable for several questions of interest, such as estimation of effective population sizes, levels of connectivity between populations, and evolutionary distinctness of certain populations. They are problematic, however, when the desired inference is to levels of genetic diversity at loci that directly influence fitness. These loci are clearly under the influence of selection and therefore influences from neutral loci may not be accurate. The issue is still under debate (Vrijenhoek, 1994; Lynch, 1996; Rodriguez-Clark, 1999) but, in general, it is reasonable to conclude that if genetic diversity is high at neutral molecular markers then genetic diversity at fitness loci also is probably high. If, however, genetic diversity is low at neutral markers then genetic diversity at fitness loci is potentially depressed, but may be sufficient to avoid inbreeding depression and loss of adaptive potential. A second way to approach the problem of extinction risk is to use genetic diversity measures as estimates of effective population size. Estimates of the rate at which new genetic variation in quantitative traits (e.g., fitness) is generated by mutation were used to suggest that populations should be maintained at a minimum effective population size of 500 individuals for long-term sustainability (Soule, 1980; Franklin, 1980). This number was estimated as the minimum needed to maintain enough quantitative genetic variation to adapt to future environmental changes. Lande (1995) subsequently suggested that the minimum effective size estimate was too small by an order of magnitude. In addition, at very low effective sizes, populations are at risk of immediate extinction from demographic effects. Therefore, estimates of genetic diversity at neutral markers should be useful indirect measures of both short and long- term sustainability because they can be used to estimate effective population sizes.

Some characteristics of genetic diversity that support its use as an indicator of ecological sustainability are summarized in Table 2-4.

Table 2-4. Rationale for indicator of ecosystem sustainability based on genetic diversity.

- Indicator of long and short-term extinction risk when combined with demographics
- Measure of potential to adapt to modified environments (e.g., susceptibility to novel pathogens)
- Amenable to temporal monitoring, trends analysis
- Can be implemented as a nondestructive measure

2.3 Measurement of Genetic Diversity

A variety of methods have been developed to measure genetic diversity within species. The oldest and most widely known is to simply measure morphological traits of individuals within populations. For traits that are determined predominantly by single genes, such as flower color or seed color in Mendel's peas, this provides a direct and straightforward measure of genetic variation. Fitness however, as well as most other morphological, physiological, and behavioral traits, is influenced by many genes, by environmental differences, and by interactions between different alleles, loci, and environments. In order to measure genetic variation of these traits, it is necessary to first control for environmental variation, a decidedly difficult task when working with natural populations.

Over the last several decades, a number of molecular markers have been developed to aid in the measurement of genetic diversity (see Avise, 1994). Since the 1970s, innumerable studies have assessed genetic diversity in the electrophoretic properties of proteins, particularly blood group proteins and enzymes (allozymes). The advantage of this system of analysis over measurement of morphological characters is that the marker patterns are almost entirely free of environmental influences and one or, at most, a few genes determine each pattern. A number of additional techniques emerged in the 1980s and 1990s for measuring genetic diversity directly at the DNA level. One of the advantages of DNA-based analysis is

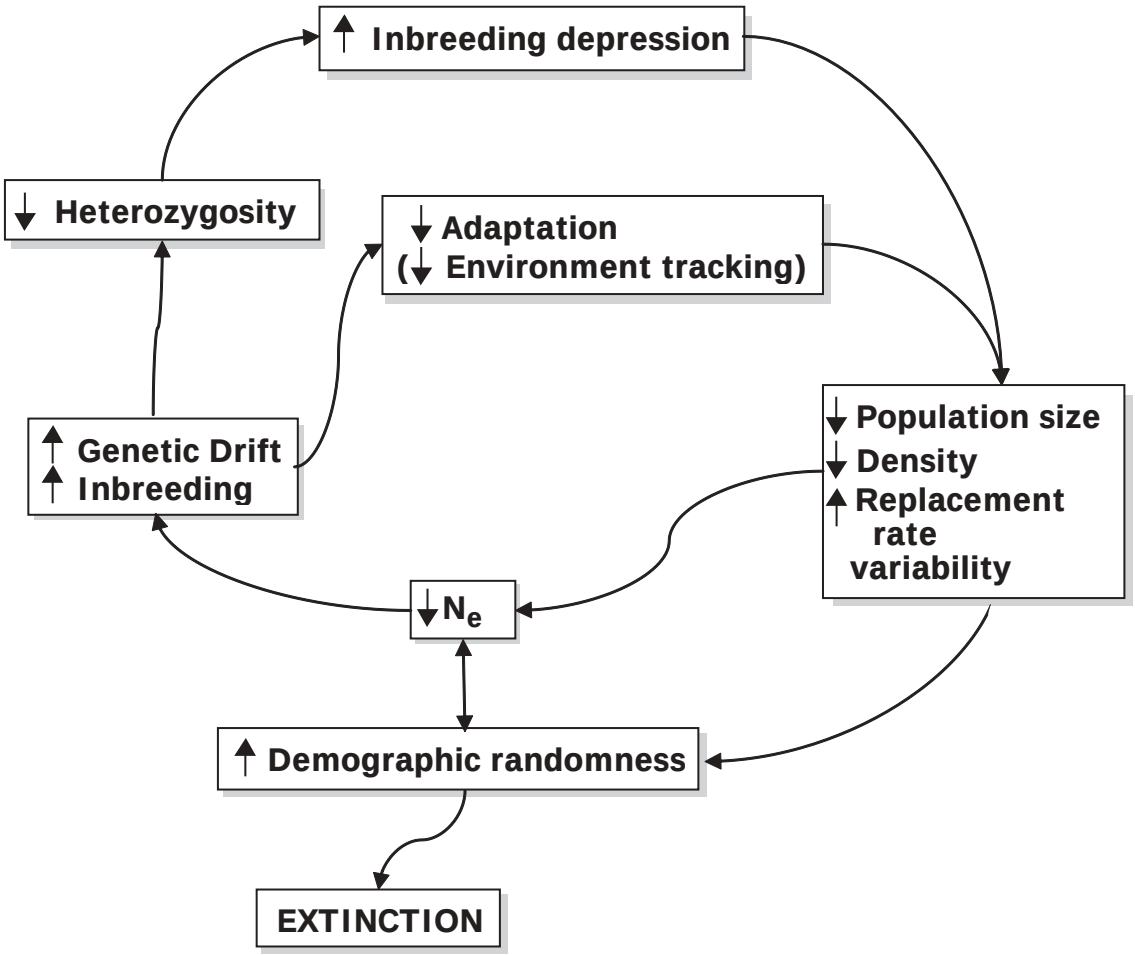

Figure 2-3. Simplified diagram of Gilpin and Soule's (1986) Extinction Vortex model. Only the adaptation and inbreeding vortices are shown. Two other vortices (population size and density) also interact with these vortices to cause population extinction. Ne: effective population size.

that genetic diversity is measured directly at the most fundamental level, before intracellular modifiers such as gene transcription, RNA splicing, and protein translation and post-translational processing can modify the pattern. Another advantage of new DNA-based methods is that essentially all of the genetic material within organisms is available for analysis. This has allowed researchers to target for analysis particular types of DNA that are most suited for answering specific questions. For example, mitochondrial DNA rarely (if ever) recombines, has a relatively high mutation rate, and is usually only transmitted from the maternal parent to offspring. This makes mitochondrial DNA particularly useful for tracking the maternal pedigree of populations back through time (e.g., for humans, back to the 'mitochondrial Eve'), and it makes genetic diversity in mitochondrial DNA particularly susceptible to the effects of small population size (genetic bottlenecks). Other types of DNA may be targeted because they have exceptionally high mutation rates and therefore generate many alleles, because they code for genes with known functions that may respond to particular environmental modifiers, or because they are believed to be selectively neutral so genetic diversity at these loci more accurately reflects evolutionary relationships between populations and species.

The mathematical foundations for measuring and interpreting genetic diversity and population genetic structure were developed in the early part of the last century. However, progress in understanding genetic processes in populations was limited until the recent explosion of molecular biological methods made it possible to accurately apply and test genetic theory in the field. Presently, genetic diversity indicators can be used to assess effective population sizes, past or present population bottlenecks, gene flow and, under some circumstances, mutation rates and selection. The availability of new types of genetic markers with different genetic properties, together with inputs from related efforts such as the human genome project and DNA forensics, has fueled a new round of mathematical development that is continuing today. As new methods to measure and interpret genetic diversity are rapidly increasing, the information about past and present population processes that ultimately can be extracted from analyses of molecular markers is almost certain to experience phenomenal growth.

3 Case Studies

Two case studies are presented in this section to demonstrate the technical feasibility of incorporating genetic diversity measurements into an ecological assessment and to help evaluate the environmental relevance of this indicator. Many variables can affect the success of a genetic diversity indicator, ranging from the quality of the genetic markers employed, to the technical abilities of the people involved, to the appropriateness of the species surveyed. The two case studies described here utilized different approaches with different technical requirements. The first study represents a large regional survey in which a large number of sites were assessed in order to create a regional genetic diversity profile for a single, moderately sensitive minnow species. The study was based on the RAPD fingerprinting technique and utilized very few samples per site. The second study included a much smaller number of sites, but evaluated more fish per site. A moderately tolerant fish was evaluated in this study with both AFLP and mitochondrial sequence data. Ideally, a genetic diversity assessment will evaluate multiple species over the study region. In fact, analyses of additional fish species are underway in both study areas; this multi-species assessment will be presented in a future document. Both case studies demonstrate the utility of integrating genetic diversity information and other ecological indicator data into a single ecological assessment.

3.1 Genetics of Central Stonerollers in The Eastern Cornbelt Plains Ecoregion

3.1.1 Background

The Central Stoneroller (Campostoma anomalum). The study organism is a small minnow in the family Cyprinidae (Figure 3-1). It is common and relatively abundant in runs or riffles over hard bottom in clear running streams throughout much of the Eastern and Midwestern USA, exclusive of Southeastern states. Stonerollers are bottom feeders, feeding primarily on algae and detritus they scrape off of rocks. Males build nests in gravel pools at the tops of riffles in which females lay between 100 and 500 eggs. Stonerollers are classified as moderately tolerant, preferring clean water in organically enriched streams with thick growths of attached algae.

Figure 3-1. The central stoneroller (*Campostoma anomalum*). (Photo courtesy of the Ohio Department of Natural Resources)

The Study Area. The Eastern Cornbelt Plains (ECBP) Ecoregion (Omernick, 1995) encompasses most of central and western Ohio as well as the central and eastern parts of Indiana (Figure 3-2). A finger of the ecoregion extends into southern Michigan. The region once was composed of tall grass prairie, eastern deciduous forest, and wetlands. Modern agriculture, industrialization, and urbanization have introduced both point source (e.g., industrial discharge, waste water treatment plants, combined sewer overflows) and non-point source (e.g., nutrient enrichment, toxic chemicals, sedimentation) stressors to the network of streams in this ecoregion. Figure 3-3 provides an aerial view of the typical ECBP landscape. The genetic study concentrated on watersheds in the eastern part of the ECBP, primarily in Ohio. Major watersheds within this region include the Scioto, Great Miami, and Little Miami, which drain into the Ohio River, and the Maumee, Sandusky, and Huron watersheds, which drain into Lake Erie.

Integration. This work was undertaken as part of a Regional Environmental Monitoring and Assessment Program (REMAP) study initiated as a collaborative effort between USEPA Region 5, the Ohio EPA and USEPA-NERL to answer questions about the overall ecological condition of the ecoregion. Specifically, the REMAP project's purpose was to assess the stream resource status and to develop biological measurements that would serve as quantitative indicators of the condition of those stream resources, the type and magnitude of stress placed on the streams, and whether the resource condition changed over time. Randomly selected sample sites along first through third order (wadeable) streams represented a variety of land uses and impact sources (Figure 3-4) present in the ecoregion.

Field sampling. Fish were collected by electroshocking (Figure 3-5) using the EMAP protocols for wadeable streams (USEPA, 1993). Central stonerollers were collected from 91 sites representing 10 large watersheds in 1995. Sample sizes at each site were small (Table 3-1) because genetic diversity sampling was not the primary purpose of the REMAP assessment. The caudal peduncle and caudal fin were removed and placed into a cryovial labeled with the site name, frozen in liquid nitrogen, and shipped back to the laboratory where the sample was stored at -80ºC.

Laboratory methods and data analysis. A brief description of laboratory and analytical procedures is provided here. We recommend that readers interested in designing a genetic study using this or related techniques consult the more detailed description in Appendix 1.

DNA was extracted from each sample using standard procedures. For this study, a genetic "fingerprint" or profile was constructed for each fish using the technique of randomly amplified polymorphic DNA (RAPD) (Williams *et al*., 1990; Welsh and McClelland, 1990). This is a relatively simple technique based on agarose gel electrophoresis of anonymous PCR (polymerase chain reaction) fragments. A drawback of the technique is that scoring of gels (detecting and sizing bands) has been found to be less repeatable than for some other methods (e.g., AFLP, microsatellite analysis). To overcome this problem, bands were scored semi-automatically using a fluorescence imaging system (Fluorimager 595, Molecular Dynamics) and dedicated image analysis software (FragmeNT, Molecular Dynamics) (Figure 3-6). Additionally, each sample was analyzed in triplicate. Bands were classified into size groups (bins) using cluster analysis. These bins were further refined by discriminant analysis of band size and intensity characteristics. In excess of 200 such bins were identified; however, 53 bins that were most repeatably scored (based on comparison of the three replicates for each individual) were selected for analysis. The genetic profile for each individual was derived from the composite information from three replicates for these 53 fragment size bins. These profiles were compared using a "similarity index" (Lynch, 1990; Leonard *et al.*, 1999). Average genetic similarities within populations provide an inverse measure of genetic diversity while average genetic similarity between two populations provides an inverse measure of the "genetic distance" between them.

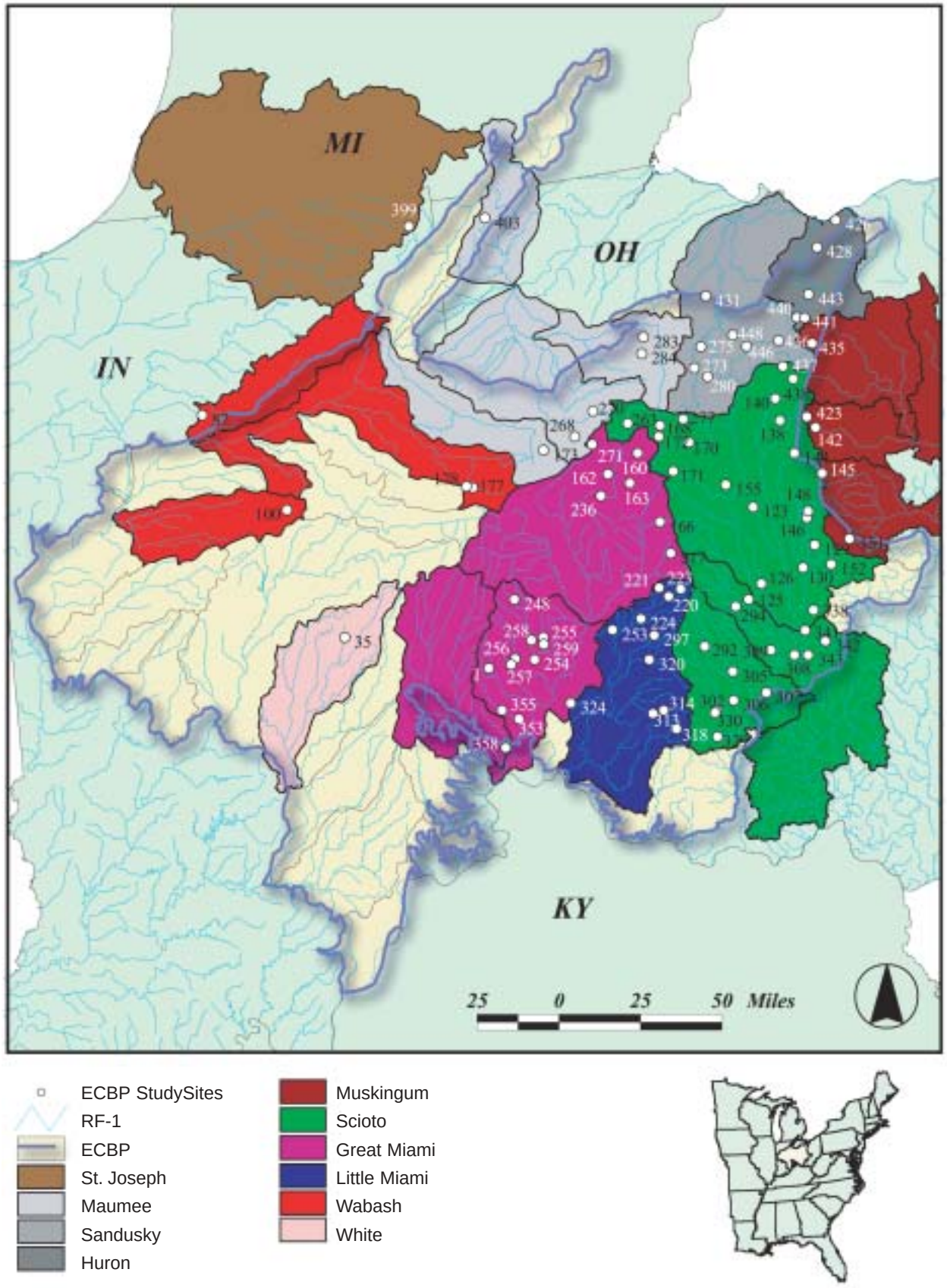

Figure 3-2. Map showing sampling locations for the study. Major watersheds are highlighted. Borders between USGS watersheds (8-digit hydrologic units or HUCs) also are indicated.

Figure 3-3. Aerial view of typical Eastern Cornbelt Plains landscape.

3.1.2 Key findings and their implications

Populations of stream fish are highly differentiated within the Eastern Cornbelt Plains Ecoregion. A common statistic used to measure how different or similar populations are within a geographic area is F_{ST} (Section 2.1). Technically, F_{ST} estimates the "inbreeding", or increased likelihood that the two copies of a gene within an individual are identical (homozygous), due to population genetic structure. An estimate of $F_{ST} = 0$ indicates that there is no evidence for geographic structure from the samples analyzed and thus they may represent a single population. An estimate of $F_{ST} = 1$ indicates that none of the populations share the same alleles at polymorphic loci. For diploid sexual species, F_{ST} values generally fall between 0 (no genetic structure and 0.3 (large genetic structure), with cases of $F_{ST} >$ 0.4 being quite unusual. For stonerollers sampled from these ECBP sites, our estimate of F_{ST} was about 0.39 (Table 3-2) so we conclude that stoneroller populations are highly structured within the study region. A nice feature of the F_{ST} statistic is that it can be used to estimate gene flow, or the average number of migrants between sites each generation. Table 3-2 shows that over the entire study area, an average of just 0.4 individuals move between populations each generation (a generation is about 2-4 years for stonerollers in this area). Of course, individuals should be much more likely to migrate to nearby streams, rather than to streams that are more distant. Looking within major watersheds, and for large watersheds within USGS 8-digit hydrologic units (HUCs), we see that the average number of migrants between sites does tend to be greater in the smaller areas. However, the pattern is not consistent for different watersheds, as some (e.g., the Scioto) show very little gene flow, on average, while others (e.g., the Wabash) show very high gene flow.

Figure 3-4. A highly modified suburban stream. Urban run-off, non-native riparian growth, bank erosion, and combined sewer overflows are common urban and suburban impacts to streams.

Figure 3-5. Backpack electroshocking for central stonerollers.

Table 3-1. Geographic location of 91 REMAP sites in the Eastern Cornbelt Plains Ecoregion from which central stonerollers were sampled. The 11-digit USGS hydrological unit code (HUC) for the sub-basin is provided, along with the REMAP site identification number, stream order classification, ecological impacts identified, Qualitative Habitat Evaluation Index (QHEI), Index of Biotic Integrity (IBI), and number of stonerollers collected. *KEY* --- RM: River mile; A: agriculture/livestock; R: riparian degradation, C: channelization; U: urban/suburban/industrial; S: waste water treatment plant or combined sewer overflow; n.c.: not calculated.

Basin	USGS HUC	Site Name	Site ID	Stream Order	Impacts	QHEI	IBI	Sample Size
St. Joseph	04050001 110	Pigeon Creek	399	3	A	80	36	10
Maumee	04100004 010	E. Br. St. Marys R. (Clear Creek)	173	3	A	57	29	10
	04100006 040	Beaver Creek	403	2	A,R,C	18.5	26	9
	04100007 010	Dry Run	268	1	A,R,C	38	46	10
	04100007 010	Auglaize River	270	3	A	72.5	44	10
	04100008 030	Eagle Creek	283	3	A	49.5	34	10
	04100008 030	Eagle Creek	284	3	A	49	24	10
Sandusky	04100011 030	Broken Sword Creek	436	3	A,R,C	34	36	7
	04100011 030	Broken Sword Creek	446	3	A	59.5	32	10
	04100011 050	Trib. to St. James Creek (RM 0.85)	280	2	A,R	33	34	9
	04100011 060	Oak Run	273	3	A,R,C	24	28	10
	04100011 060	LittleTymochtee Creek	275	3	A	52.5	20	8
	04100011 070	Sycamore Creek	448	3	A	62.5	42	10
	04100011 100	E. Br. Wolf Creek	431	3	A	54.5	28	10
Huron	04100012 010	Marsh Run	440	3	A	51	34	10
	04100012 010	Walnut Creek	443	3	A	69	46	10
	04100012 030	Trib. to trib. to Huron R.	428	1	U,R,C	30.5	28	9
	04100012 040	Chappel Creek	426	2	none	49	36	10
Muskingum	05040002 010	Trib. to Black Fk. Mohican R. (RM 54.45)	435	2	A	58	40	9
	05040003 010	Kokosing River	142	3	none	42.5	44	10
	05040003 010	Kokosing River	423	2	A	73	53	10
	05040006 010	Otter Fk. Licking R.	145	2	R,C	26	36	10
	05040006 040	Trib. to S. Fk. Licking R.	151	2	none	61	38	10
Scioto	05060001 010	Taylor Creek	168	2	A	71.5	36	10
	05060001 010	Trib. to Taylor Creek (RM 7.60)	172	1	A	31	28	3
	05060001 010	Trib. to Scioto River (RM 227.76)	263	2	A	32	36	9
	05060001 020	Rush Creek	170	3	A	67.5	37	9
	05060001 030	Panther Creek	277	2	A	62.5	40	10
	05060001 070	Grassy Run	155	2	A,C	27	26	10
	05060001 070	Trib. to Mill Creek (RM 41.24)	171	3	A	58.5	40	9
	05060001 080	Eversole Run	123	3	none	71.5	48	10
	05060001 090	Flat Run	140	3	none	79	43	9
	05060001 090	Trib. to Olentangy R. (RM 76.95)	437	1	A,R,C	24.5	30	8
	05060001 090	Rocky Fork	438	3	R	53.5	32	10
	05060001 100	Shaw Creek	138	3	A,C	44	38	10
	05060001 140	Blacklick Creek	129	3	U,S	63.5	48	10
	05060001 140	Blacklick Creek	130	3	U,S	75	38	6
	05060001 140	Rocky Fk. Big Walnut	146	2	U	67.5	48	3
	05060001 140	Rocky Fk. Big Walnut	148	2	A	67	36	8
	05060001 150	Bunker Run	144	3	none	68.5	54	10
	05060001 170	(none provided)	152	2	n.c	n.c	n.c	10
	05060001 180	Lick Run	338	2	A	54	46	10
	05060001 220	Hellbranch Run	126	3	U,S	75	45	18
	05060002 010	Yellowbud Creek	308	3	A	80.5	56	20

(continues on next page)

Table 3-1. (Continued)

Basin	USGS HUC	Site Name	Site ID	Stream Order	Impacts	QHEI	IBI	Sample Size
	05060002 010	Hargus Creek	341	3	U,S	58	36	10
	05060002 010	Scippo Creek	342	3	A,S	73.5	54	10
	05060002 010	Congo Creek	343	3	A	82	54	10
	05060002 030	Opossum Run	125	2	R	72.75	47	10
	05060002 030	Bradford Creek	294	3	A	84.5	55	9
	05060002 040	Hay Run	309	3	A	63.5	48	10
	05060003 020	Sugar Creek	292	3	A,R	43	50	10
	05060003 040	Bull Creek	302	2	none	76	54	10
	05060003 040	Hardin Creek	330	2	A	71	50	10
	05060003 050	Paint Creek	305	3	A	76.5	37	3
	05060003 050	Sugar Run	306	2	A	62	48	10
	05060003 060	Clear Creek	329	1	A	50	40	7
	05060003 090	Trib. to Little Creek (RM4.22)	307	2	none	53	44	10
Great Miami	05080001 010	Trib. to S. Fk. Great Miami R. (RM 5.27)	160	3	A	76.5	52	9
	05080001 020	Willow Creek	271	2	A,R,C	26	46	10
	05080001 030	Brandywine Creek	162	2	A,R,C	30.5	38	9
	05080001 030	Blue Jacket Creek	163	2	A	32	20	8
	05080001 040	Little Indian Creek	236	2	A	75	50	10
	05080001 150	Kings Creek	166	3	A	58	38	10
	05080001 170	E. Fk. Buck Creek	217	2	A,R,C	49	40	19
	05080002 030	Twin Creek	248	2	A	59	48	10
	05080002 040	Trib. to Twin Creek (RM 18.29)	254	2	A	54	50	7
	05080002 040	Toms Run	255	2	A	62	n.c	10
	05080002 040	Bantas Fork	258	3	A	75	42	10
	05080002 040	Toms Run	259	2	none	82	42	10
	05080002 050	Millers Creek	324	2	none	65.5	46	10
	05080002 060	Sevenmile Creek	256	3	A	73.5	46	9
	05080002 060	Paint Creek	257	3	A	74	42	9
	05080002 070	Fourmile Creek	1	3	A,R,C	29	24	8
	05080002 080	Indian Creek	353	3	S	71.5	45	7
	05080002 080	Indian Creek	355	3	none	80.5	40	9
	05080003 080	Trib. to Dry Fk. Whitewater R. (RM 6.73)	358	1	none	68.5	46	10
Little Miami	05090202 010	Little Miami River	220	3	A	75.5	38	10
	05090202 010	Trib. to N. Fk. L. Miami R. (RM 5.60)	221	2	A,R,C	23.5	28	4
	05090202 010	Lisbon Fork	223	2	A,R	54	46	10
	05090202 020	Clark Run	224	2	A	58	38	10
	05090202 020	Little Beaver Creek	253	2	U,S	91	38	10
	05090202 040	Painters Creek	320	2	A	71.5	46	10
	05090202 050	N. Br. Caesar Creek	297	3	A,R	49	44	7
	05090202 080	E. Fk. Todd Fork	313	3	A	60	50	10
	05090202 080	E. Fk. Todd Fork	314	3	A	54.5	46	10
	05090202 100	Turtle Creek	318	3	S	58.5	54	9
Wabash	05120101 010	(none provided)	177	3	n.c	n.c	n.c.	10
	05120101 010	Wabash River	178	3	A	66	31	9
	05120104 070	W Br Twelvemile Cr.	87	2	A	53.5	31	10
	05120107 010	Middle Fork Creek	100	1	A,C	58	40	10
White	05120204 020	Sixmile Creek	35	2	None	60	48	5

Populations of stream fish are geographically structured among and within watersheds. Another way to look at these data is to examine the patterns of relatedness between each of the sampling sites. One can think of the populations as connected in a phylogenetic tree-like pattern, with the caveat that, unlike relationships between different species, the patterns of relationships are determined by both evolutionary descent and ongoing gene flow. Figure 3-7 shows a tree diagram constructed from a matrix of genetic distance between each of the stoneroller collection sites using the neighbor joining method. Populations in the same basin are colored similarly. The genetic distance between any two populations is represented as the total length of the branch between them. Two things are immediately obvious from the figure. First, groups of populations in the same watershed tend to cluster together on the tree (genetically similar). Second, some watersheds have more than one cluster of genetically related populations while some clusters include more than one watershed. For example, the Scioto basin (sites colored green in Figure 3-7) is clearly separated into two highly distinct genetic groups. Examination of the geographic location of sites (Figure 3-2) indicates that the smaller genetic group is composed of populations located in the extreme northern part of the basin. All are from a single USGS watershed (HUC 50600001), but other populations from this same watershed are from the second genetic group. In contrast, populations in the Little Miami (sites shown in blue) and Great Miami (sites shown in purple) watersheds are not highly distinct from each other.

Another way to visualize genetic relationships is with multidimensional scaling (MDS). In MDS analysis, the relationships between each population and all other populations are represented in two dimensions in order to more easily observe patterns. In Figure 3-8, the separation between the two genetic groups in the Scioto basin is evident, as is the lack of strong separation between the Little Miami and Great Miami

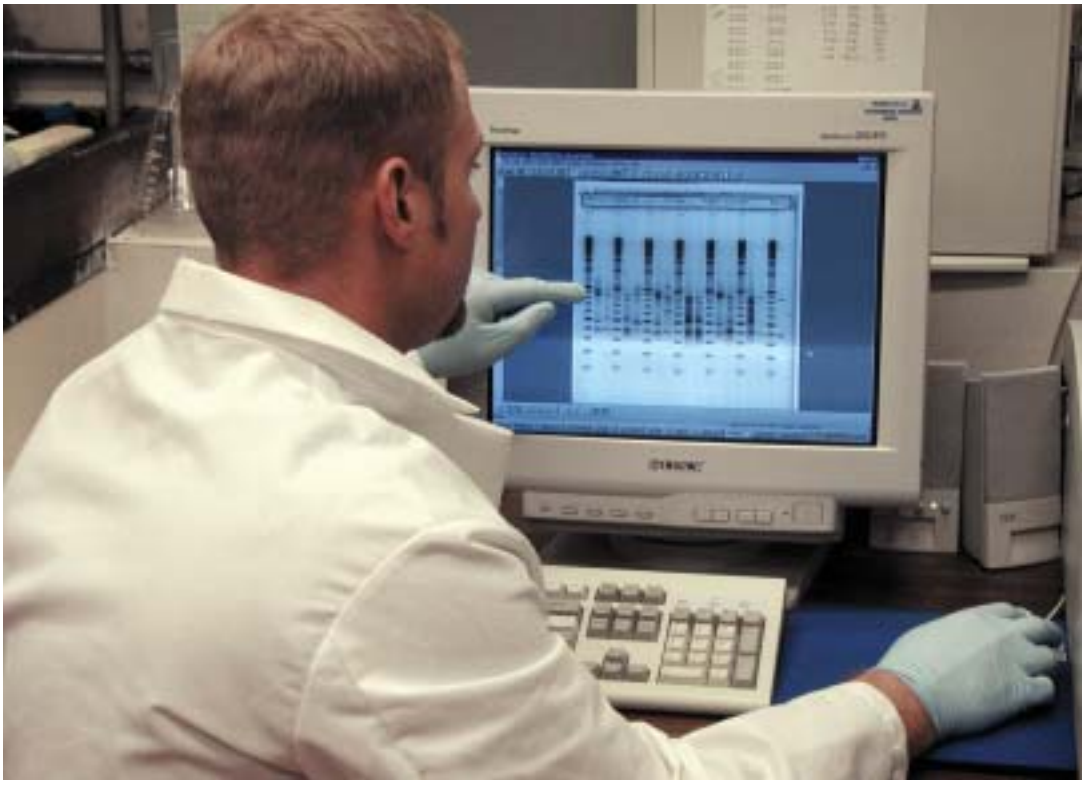

Figure 3-6. Interpretation of marker patterns is aided by gel image analysis software

basins. However, it also is clear that many of the populations from streams in the Erie drainage are genetically very similar, and are not strongly differentiated from streams in the Wabash basin. Based on this genetic analysis, we can define at least 5 large genetic groups within the ECBP ecoregion.

The major significance of this analysis is that understanding the genetic structure of stream fish populations is requisite to drawing conclusions about the status of those species and, by extension, the habitats that support them. Populations within each of the five genetic groups are likely to have some degree of genetic interdependence among them, either due to ongoing gene flow or recent separation. These populations are likely to share common genetic traits (e.g, susceptibilities, behaviors) and have some degree of demographic connectivity. Populations in different genetic groups, however, are likely to be highly independent, demographically, genetically, and evolutionarily. These genetic groups, therefore, are appropriate units for performing ecological analyses if the assessment question concerns the current and future status of stoneroller populations. If central stoneroller population structure parallels that of other minnow species, the geographic boundaries between these genetic groups delineate appropriate assessment units for minnows in general. It should be noted that the boundaries of these genetic groups do not compare well to watersheds defined by 8-digit USGS hydrologic unit codes (HUCs), although these are the typical assessment units in current use.

Genetic diversity differs among populations and among genetic groups. A useful method for measuring genetic diversity with DNA fingerprint data is to estimate the average genetic similarity of individuals within populations (Sw; Lynch, 1990). This is an inverse measure of genetic diversity, so populations with high genetic similarity are less diverse. Average genetic similarity within these populations ranged from 58% to 84%. A somewhat surprising result of this analysis was that genetic diversity within populations differed greatly among the five genetic groups (Figure 3-9). Populations in the Great Miami and Little Miami basins (genetic groups 1 and 2) were slightly more genetically diverse (less average genetic similarity within populations) than populations in the lower Scioto Basin (genetic group 3) and were much more diverse than populations in the Erie drainage (genetic group 5). These differences in genetic diversity at geographically broad scales are not likely to reflect different anthropogenic impacts. Instead, they probably reflect evolutionary differences in the amount of diversity within these groups. The general trend of decreasing diversity with increasing latitude may reflect residual effects of the Wisconsin glaciation, which would have wiped out fish populations over most of the study area. Regardless of how the differences arose, these results suggest that the future sustainability of populations in the Little Miami and Great Miami basins is much less in question than the sustainability of Erie drainage populations.

Table 3-2. Summary of population genetic structure analysis.

Geographic grouping	Populations	F_{ST}	Average number of migrants per generation (N_em)	Significance level
Overall	91	0.388	0.4	< 0.001
Maumee	6	0.046	5.2	0.001
Sandusky	7	0.033	7.3	0.16
Huron	4	0.026	9.4	0.05
Muskingum	5	0.298	0.6	< 0.001
Scioto	34	0.325	0.5	< 0.001
HUC 5060001	20	0.360	0.4	< 0.001
HUC 5060002	7	0.065	3.6	< 0.001
HUC 5060003	7	0.096	2.4	< 0.001
Little Miami	10	0.148	1.4	< 0.001
Great Miami	19	0.110	2.0	< 0.001
HUC 5080001	7	0.077	3.0	< 0.001
HUC 5080002	11	0.120	1.8	< 0.001
Wabash	4	0.006	41.4	0.31

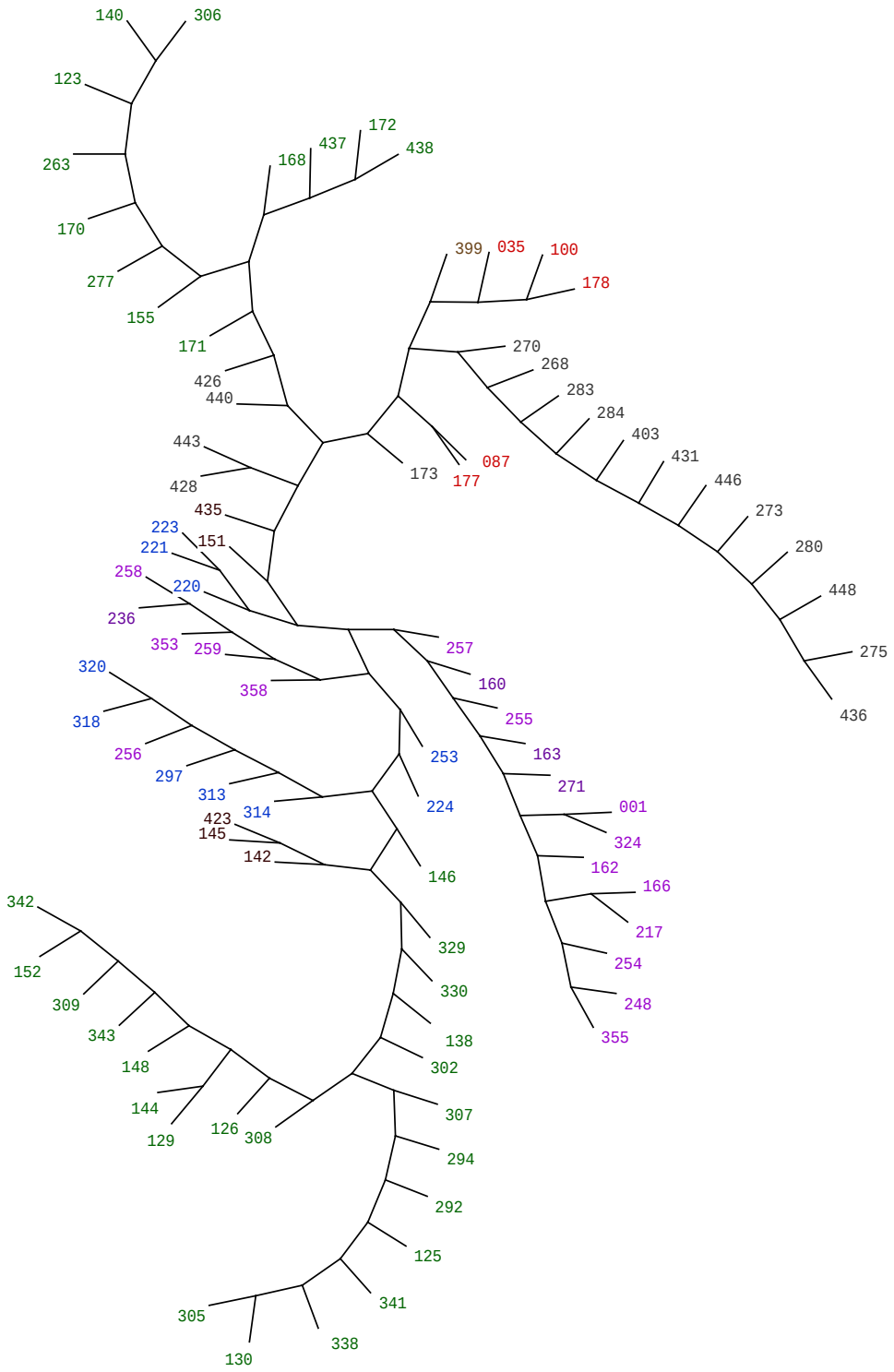

Figure 3-7. Tree diagram depicting the estimated genetic relationships between populations at each site. This is an unrooted tree which was made by employing the neighbor-joining algorithm to impose a tree structure on a matrix of Nei's genetic distances between each site. Site IDs are color coded to match the map in Figure 3.2.

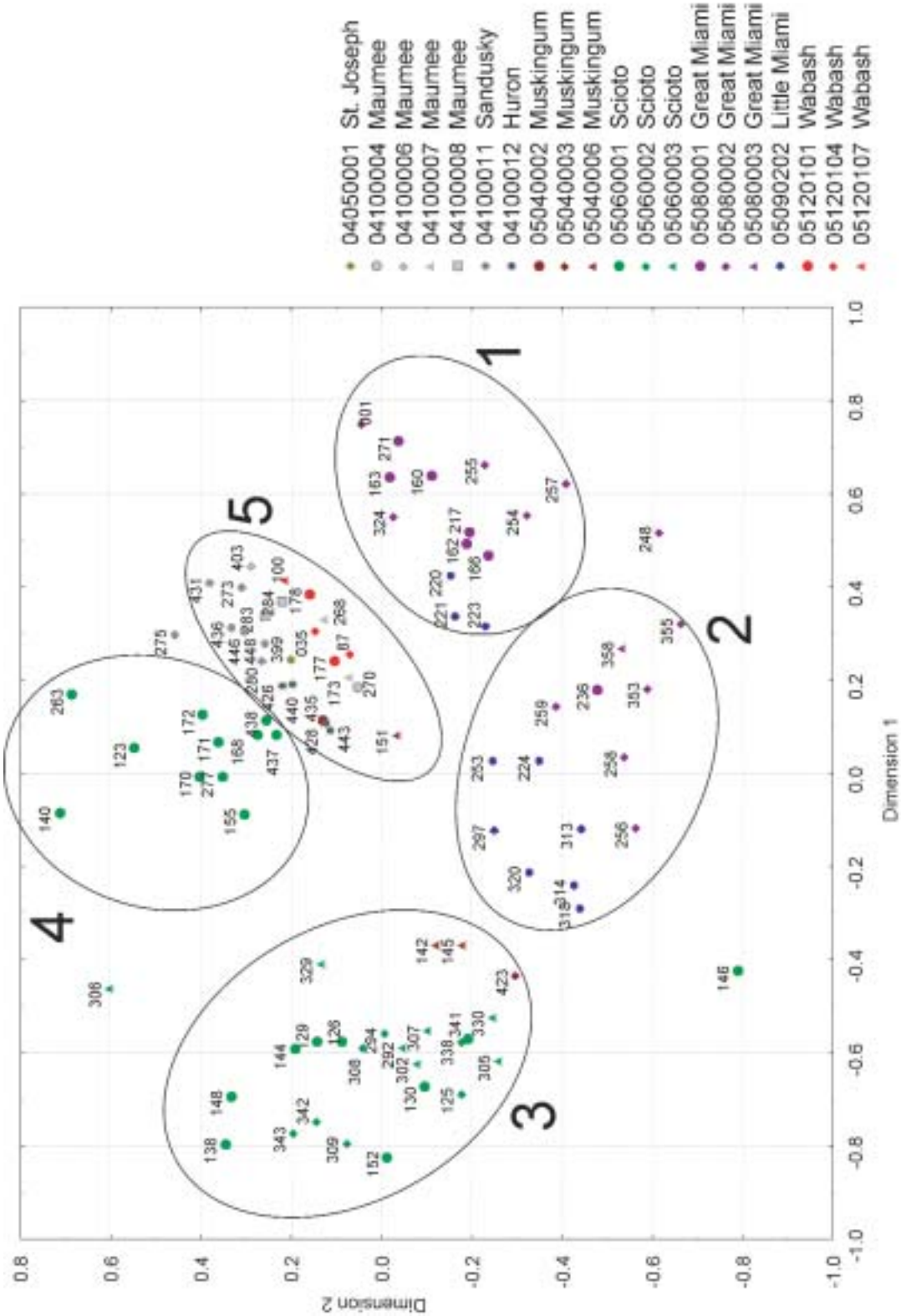

Figure 3-8. Multidimensional scaling (MDS) analysis of relationships between stonerollers at the different sampling sites. Sites that are close together in this two-dimensional space are more genetically similar. Circles were drawn around five genetically well-defined groups following the MDS analysis.

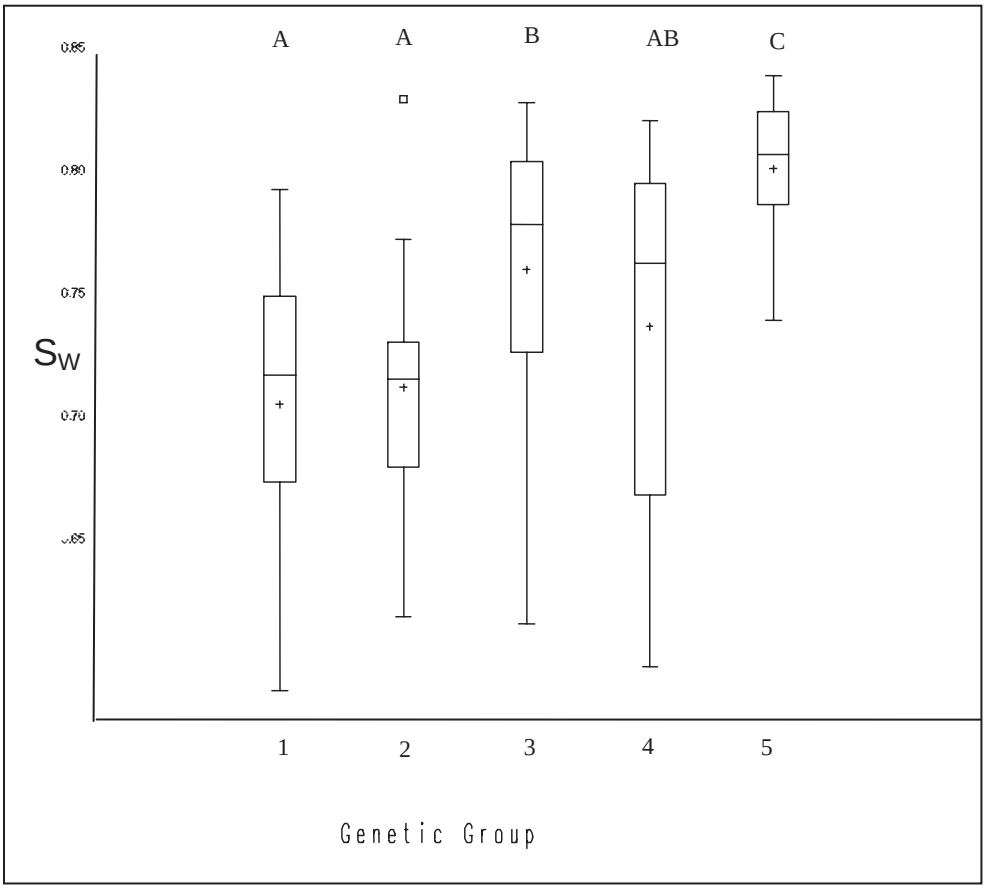

Figure 3-9. Box and whisker plot of genetic similarity within populations (S_W) for each of the five genetic groups identified in Figure 3-8. Groups with low average genetic similarity have higher genetic diversity. Groups with different letters have significantly different levels of genetic diversity ($p < 0.05$, Duncan's multiple range test).

Genetic diversity within populations is affected by environmental condition.

Correlations between the average genetic similarity within populations and various environmental measures are presented in Table 3-3. The environmental measures include the Qualitative Habitat Evaluation Index (QHEI) and 8 metrics that are used to create the index. Other measures include tissue metabolite assays for benzo(a)pyrene (BAP)-type compounds (generally associated with combustion by-products) and naphthalene (NAPH)-type compounds (associated with oil contamination), and EROD assays which measure enzyme activity induced by planar xenobiotics such as halogenated hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). The raw correlations were generally small. Among the QHEI metrics, only the overall QHEI index and the cover and pool metrics were significant, although all correlations with genetic similarity were negative, as would be expected (larger values of the metrics indicate better environmental quality).

Table 3-3. Spearman (rank) correlations between average genetic similarity within populations (Sw) and various environmental measures.

Environmental Variable	N[1]	Mean	Std. Dev	Spearman Correlation
Qualitative Habitat Evaluation Index	84	58.33	16.85	-0.229*
Substrate QHEI metric	84	12.20	5.31	-0.166
Cover QHEI metric	84	11.53	3.99	-0.216*
Channel QHEI metric	84	13.02	4.27	-0.160
Riparian QHEI metric	84	5.21	1.96	-0.022
Pool QHEI metric	84	6.16	2.64	-0.269*
Riffle QHEI metric	84	2.50	1.95	-0.135
Gradient QHEI metric	84	7.71	2.20	-0.121
Gradient (m/km)	84	3.02	2.98	-0.101
Average sampling depth (cm)	83	34.58	17.96	-0.302**
Latitude	86	40.17	0.62	0.492***
Longitude	86	-83.63	0.77	0.268*
Stream Order (first-third)	86	2.47	0.63	0.088
Watershed Area (hectares)	86	8361	3844	-0.050
Watershed perimeter (km)	86	54.62	16.82	-0.050
Elevation (meters)	86	279.86	40.09	-0.170
BAP (µg/mg protein)	78	0.15	0.11	-0.088
NAPH (µg/mg protein)	78	27.77	10.19	0.104
EROD (pmol/min/mg protein)	76	6.96	7.84	0.070

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

[1]Sample sizes vary because not all environmental measures were available for all sites.

As an exploratory analysis, variables that suggested a strong association with genetic similarity in simple correlation analyses were included in a linear model to examine their explanatory power in determining average genetic similarity [S_w was transformed ($S_w/(1-S_w)$) to approximate normality]. Both quantitative variables (Table 3-3) and categorical variables (genetic group and the impact factors from Table 3-1) that were significant at the $p < 0.1$ level were included in the initial model. The least significant effects were eliminated in a stepwise fashion until only effects that were significant at the $p < 0.05$ level were left in the final model.

The final model (Table 3-4) was highly significant ($F_{8,74} = 10.8$, $p < 0.0001$) and explained about half of the variation in S_w ($R^2 = 0.53$). Significant effects in the final model included three impact factors: Urban, Riparian, and Channelization, along with genetic group and average stream depth at the sampling location. Latitude, though highly significant in the correlations analysis, was not significant in the linear model because it was strongly associated with genetic group. It is interesting that the riparian and channelization impact factors were significant while the QHEI riparian and channel metrics were not. The explanation for this may be a nonlinear response of genetic diversity to habitat degradation, as only highly affected sites were classified as having impacts to the riparian zone or channelized habitat. Stream depth may be significant as it is an indicator of stream size, and thus the carrying capacity of the habitat.

Table 3-4. Final model explaining genetic similarity within populations following stepwise elimination of non-significant effects.

Source	Degrees of Freedom	Mean Square	F Value	Probability
Impact factor - Urban	1	5.369	11.20	0.001
Impact factor - Riparian	1	4.882	10.19	0.002
Impact factor - Channelization	1	2.452	5.12	0.027
Major Genetic Group	4	6.311	13.17	< 0.001
Depth (covariate)	1	1.974	4.12	0.046

We can use these relationships to derive a more precise ecological indicator based on genetic diversity. Specifically, the deviation of the estimated genetic diversity within each site from its predicted value based on historical biogeographic factors (genetic group) and habitat carrying capacity (stream depth) indicates the relative genetic health of the population. Figure 3-10 shows a representation of this type of analysis, in which the corrected genetic diversity at each site is classified as low, moderate, or high. As with any ecological indicator, these results should be interpreted cautiously and only in relation to other indicators of ecological condition.

Genetic diversity is weakly associated with other indicators of environmental condition. In addition to indicators that might directly affect genetic diversity, other environmental variables were measured that are not expected to affect genetic diversity directly, but might be similarly affected. If measures of genetic diversity are redundant with other indicators, then direct measures of genetic diversity may be unnecessary. Table 3-5 demonstrates that genetic similarity within central stoneroller populations is significantly correlated with two common indicators of environmental condition, the Index of Well Being and Index of Biotic Integrity, but the correlation coefficients are small. Genetic similarity also is correlated with several other environmental measures, however these correlation coefficients also are small. We interpret these results to signify that the genetic similarity measure for central stonerollers is related to but not highly redundant with these other indicators of ecological condition.

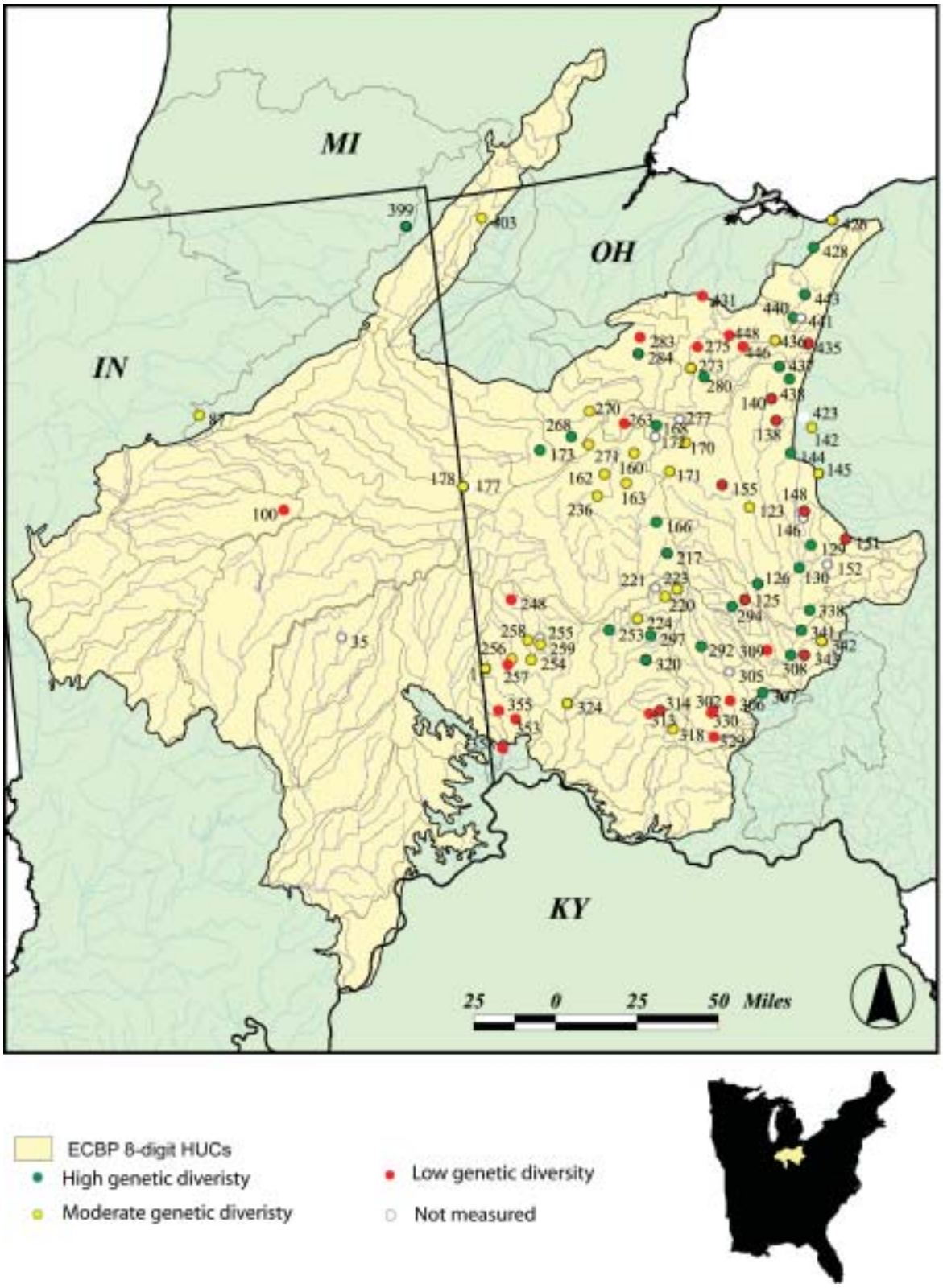

Figure 3-10. Map depicting the relative genetic diversity at each site after correcting for genetic group and average stream depth at the sampling site. Green: highest one third of sites; yellow: middle third of sites; red: lowest third of sites

Table 3-5. Spearman rank correlations of condition indicators with average genetic similarity.

Variable	N	Mean	Std.Dev.	Spearman Correlation
Number of fish (per 300 m)	83	1454.00	1108.00	-0.235*
Weight of fish (kg/300 m)	83	14.73	15.94	-0.243*
Total fish species	83	17.88	6.13	-0.310**
Shannon Diversity Index (on number)	83	1.93	0.35	-0.025
Shannon Diversity Index (on weight)	83	1.79	0.35	-0.078
Index of Well Being	83	8.32	1.12	-0.228*
Index of Biotic Integrity	83	40.71	8.58	-0.287**
Number of sunfish species	83	2.40	1.35	-0.168
Number of darter species	83	3.57	1.80	-0.163
Number of sucker species	83	1.98	1.20	-0.270*
Number of Cyprinid minnows	83	7.03	2.13	-0.321**
Number of intolerant spp.	83	0.76	1.18	-0.130
Percent tolerant fish	83	45.79	20.81	0.137
Percent top carnivores	83	1.72	2.37	-0.139
Percent omnivores	83	18.10	13.86	-0.096
Percent fish with anomalies	83	0.28	0.65	0.049
Number of sensitive or moderately sensitive species	83	822.59	832.45	-0.260*
Number of sensitive species	83	4.31	3.47	-0.306**
Percent simple lithophils	83	27.63	14.98	-0.047
Number simple lithophils	83	6.34	2.94	-0.302**

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

3.2 Genetics of Creek Chubs in a Mining-Impacted Region

3.2.1 Background

The Creek Chub (*Semotilus atromaculatus*). Like the central stoneroller, this organism is a minnow in the family Cyprinidae (Figure 3-11). It is common in small to moderate size hard bottom streams from the Great Plains to the Atlantic coast. Creek chubs are omnivorous, eating primarily insects and plant material. Some adults can reach substantial size (250-300 mm) and become significant fish predators. Males build and defend nests in gravel beds where there is moderate current. Females lay a clutch of approximately 25-50 eggs in the nest, which is covered with gravel by the male but not defended. Creek chubs are fairly tolerant of turbidity and pollution.

Figure 3-11. The creek chub (*Semotilus atromaculatus*) (Photo courtesy of the Ohio Department of Natural Resources)

The Study Area. The EPA initiated a regional demonstration project in the mid 1990s to evaluate the condition of wadeable streams in the Middle Atlantic region of the United States. For this pilot study, we focused on a smaller region within Pennsylvania and West Virginia underlain by coal-bearing geology and for which coal mining operations are historically known. Five watersheds, as determined by USGS 8-digit hydrologic units (HUCs) were represented: Upper Ohio, Connoquenessing, Upper Ohio-Wheeling, Little Muskigum-Middle Island, and Little Kanawha (Figure 3-12); all are within the Western Allegheny Plateau ecoregion. While this region is primarily forested, between 14 and 28% of the stream length in each of the watersheds studied in this pilot has agricultural land cover in the riparian zone (USEPA, 1997).

Integration. This study was performed in conjunction with the EPA's Mid-Atlantic Integrated Assessment (MAIA) project, under the Environmental Monitoring and Assessment Program (EMAP). EMAP is a research program charged with developing the tools necessary to monitor and assess the status and trends of national ecological resources and typically employs a range of ecological indicators in order achieve this purpose.

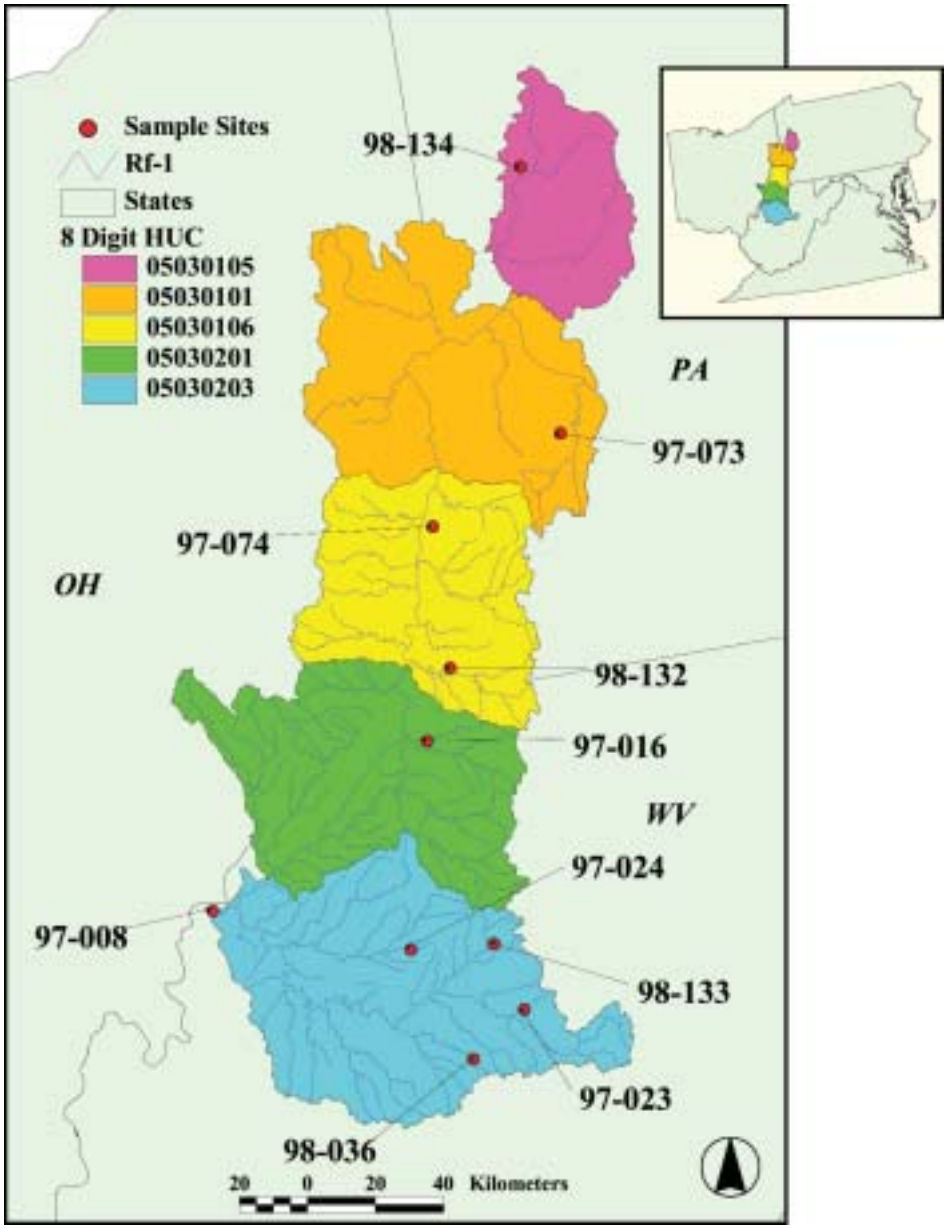

Figure 3-12. Map indicating the location of sample sites within the five watersheds studied. The USGS 8-digit hydrologic units are indicated. These watersheds are (from North to South): Upper Ohio, Connoquenessing, Upper Ohio-Wheeling, Little Muskigum-Middle Island, and Little Kanawha. All sites fall within Pennsylvania and West Virginia.

Field sampling. Fish were collected using the EMAP protocols for wadeable streams (USEPA, 1993). Sites were chosen probabilistically in order to establish the overall condition of first through third order streams in the area. Measurements of physical habitat, water and sediment chemistry, and biotic assemblage structure were used to develop quantitative indicators of the condition of stream resources and the types and magnitudes of stresses placed on streams. Samples of between 9-28 creek chubs were collected in 1997-98 from 10 sites within the five watersheds (Table 3-6).

Laboratory methods and data analysis. A brief description of laboratory and analytical procedures is provided here. We recommend that readers interested in designing a genetic study using this or related techniques consult the more detailed description in Appendices 2 and 3.

Total genomic DNA was extracted from the caudal fin of each sample using standard procedures. For this case study, two analytical techniques were used: a genetic "fingerprint" was constructed for each fish based on its nuclear DNA characteristics and a portion of its mitochondrial DNA was sequenced. The DNA fingerprint was produced using the technique of amplified fragment length polymorphism (AFLP; Vos *et al.* 1995). This is a relatively simple technique based on gel electrophoresis of anonymous PCR fragments. Twenty percent of the creek chub samples were analyzed in duplicate. The genetic profile for each individual was derived using the presence or absence of 109 polymorphic AFLP markers. As in the

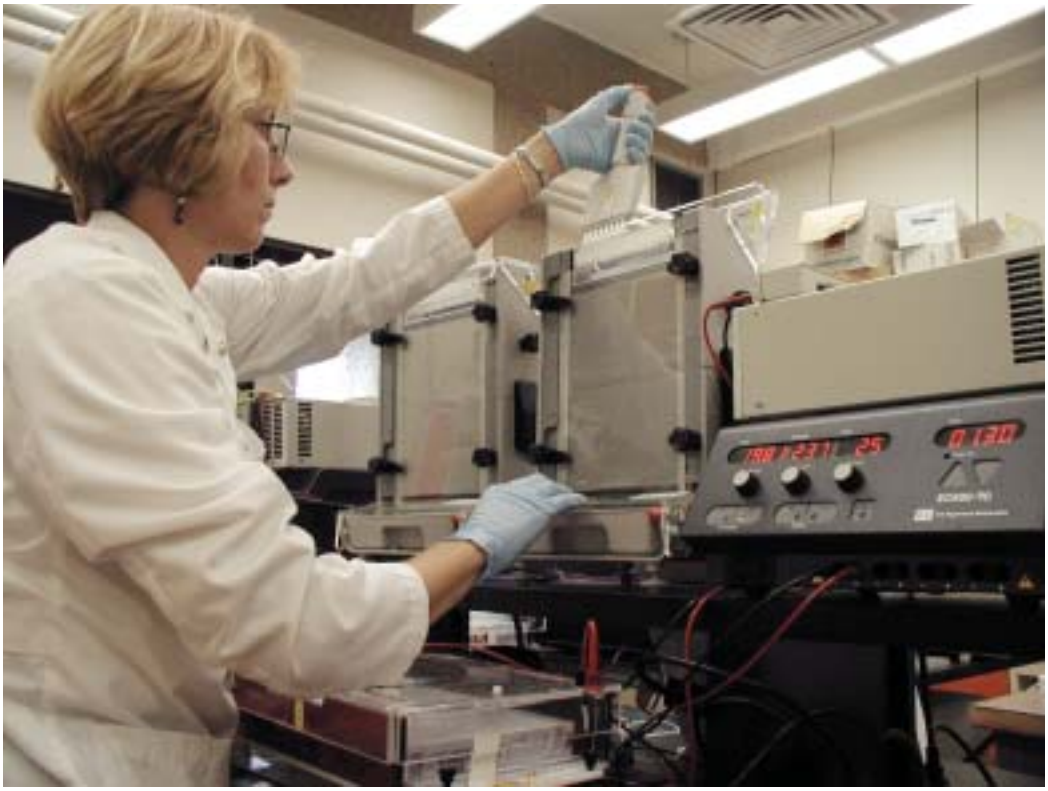

Figure 3-13. Manual gel loading of samples in an acrylamide gel for electrophoresis.

first case study, these genetic profiles were compared using a similarity index approach (Lynch, 1990; Leonard *et al.*, 1999). A 590 base pair (bp) portion of the cytochrome B gene was sequenced in order to provide a measure of genetic diversity in the mitochondrial genome.

To provide an ecological context for the genetic diversity patterns, a set of 25 environmental measures also were examined. These environmental factors were analyzed and reduced to six independent "factors" that explained most of the environmental differences between sites using principal components analysis. Reducing the number of environmental variables and interdependence among them is very helpful in understanding the difference between sites and performing statistical analyses. Relationships between genetic diversity and these environmental factors were explored with multiple regression analysis.

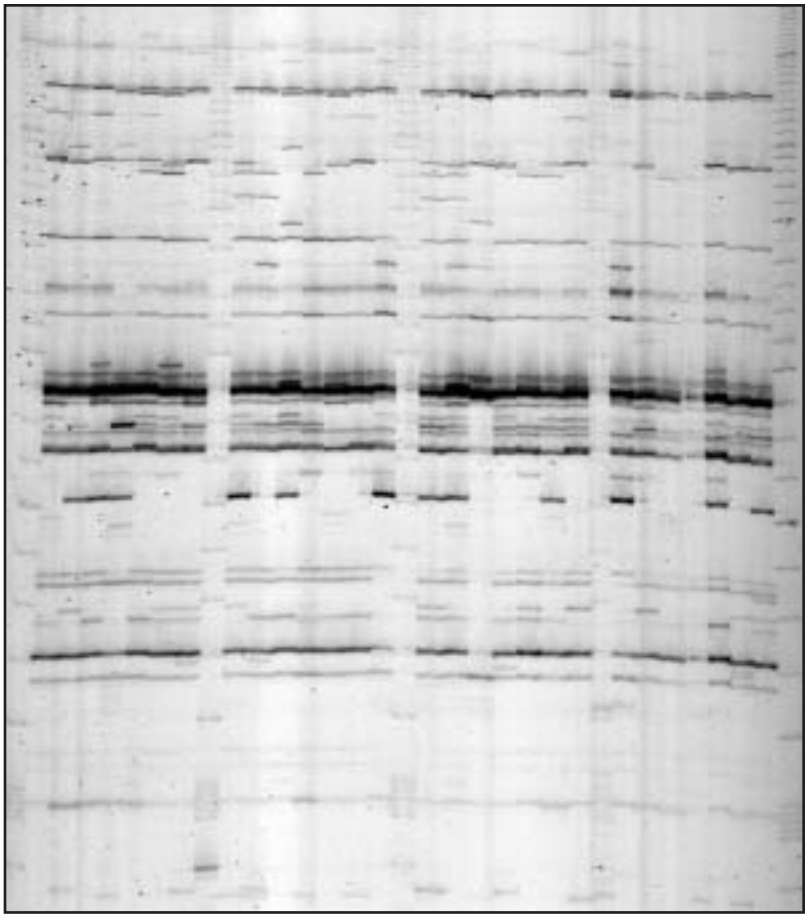

Figure 3-14. An AFLP fingerprint gel. The first lane and every 8th lane following it contains a 10 bp ladder to aid in sizing the fragments. Lanes between these ladders contain AFLP fingerprints for different creek chub samples. Polymorphisms are scored as the presence or absence of individual fragments in each lane.

3.2.2 Key Findings and their Implications

Mitochondrial DNA differences between creek chubs have a strong spatial component. Among the DNA samples sequenced, 27 unique types of mitochondrial DNA (mitochondrial haplotypes) were identified. By inspecting the pattern of mutational differences between these different forms, a network or phylogenetic tree can be constructed that shows how one mitochondrial form has evolved into another through time. One way to represent this data visually is in the form of a minimum spanning network (Figure 3-15). In the figure, each colored circle represents one of the mitochondrial DNA haplotypes identified. The frequency of the mitochondrial type in the overall sample is proportional to the area of the circle, and its spatial distribution is represented by different colors. Lines connecting circles represent mutations that differentiate different mitochondrial haplotypes; black dots represent haplotypes that are inferred to exist (or have existed) but were not observed. The figure shows that two common, presumably ancestral haplotypes were identified, along with many related but infrequent haplotypes. The five populations from the four northern watersheds have mitochondria that are from one group, while the five populations from the Little Kanawha watershed (HUC 05030203) were predominantly of the other

Table 3-6. Geographic location of 10 sites within the Western Allegheny Plateau ecoregion from which creek chubs were sampled. The name and 8-digit USGS hydrological unit code (HUC) for each watershed is provided, along with the individual stream/site name, stream ID, stream order classification, and number of creek chubs used in nuclear (AFLP) and mitochondrial genome analysis. Colors correspond to those of the watersheds depicted in Figure 3-12.

Watershed	USGS HUC	*Site Name*	MAIA Stream ID	Stream Order	Sample Size for AFLP analysis	Sample Size for mitochondrial analysis
Upper Ohio	05030105	Taylor Run	98-134	2	27	27
Connoquenessing	05030101	Millers Run	97-073	3	14	14
Upper Ohio-Wheeling	05030106	Short Run Creek	97-074	3	14	14
	05030106	Upper Bowman Run	98-132	2	28	25
Little Muskigum-Middle Island	05030201	Little Fishing Creek	97-016	3	9	8
Little Kanawha	05030203	Neal Run	97-008	1	13	13
	05030203	Spruce Creek	97-024	3	14	13
	05030203	Walnut Fork	98-133	2	27	26
	05030203	Oil Creek	97-023	3	13	13
	05030203	Left Fork Steer Creek	98-036	3	15	13

mitochondrial group. It is interesting to evaluate this geographically structured genetic pattern (or "phylogeographic" pattern) in relation to the geologic history of this area. Prior to the Pleistocene glaciations, streams in the four northern watersheds drained into the Great Lakes/Laurentian basin while streams in the Little Kanawha watershed drained into the Mississipi River via the ancestral Teays River. Current patterns of mitochondrial genetic differentiation appear to reflect this historical structure.

A useful way to evaluate the geographic structure of mitochondrial DNA difference is with an analysis of molecular variance (AMOVA, Excoffier *et al*, 1992), which is analogous to the common analysis of variance. Using this method, we can determine the proportion of all major variation that is due to differences among regional groups (the northern four watersheds vs. the southernmost watershed), differences among populations within watersheds, and differences among individuals within populations. The results of this analysis indicate that 65% of all mitochondrial variability is associated with the regional differences, while 7% is associated with differences among populations within watersheds. Just 28% of mitochondrial variability occurs among individuals within populations (Figure 3-16). This information can be used to derive an estimate of F_{ST} of 0.72, indicating that mitochondrial haplotypes of creek chubs are very highly differentiated in the study area.

Creek chub populations are strongly differentiated in the nuclear genome, though the genetic structure is not as strong as for mitochondrial DNA. As was shown for stoneroller DNA fingerprints in the first case study, the patterns of genetic similarities within and among populations can be converted into genetic distances and subjected to a standard cluster analysis to reveal the evolutionary relationships among populations. Figure 3-17 shows the pattern of evolutionary relationships among these ten populations. The length of the branch between any two populations is roughly

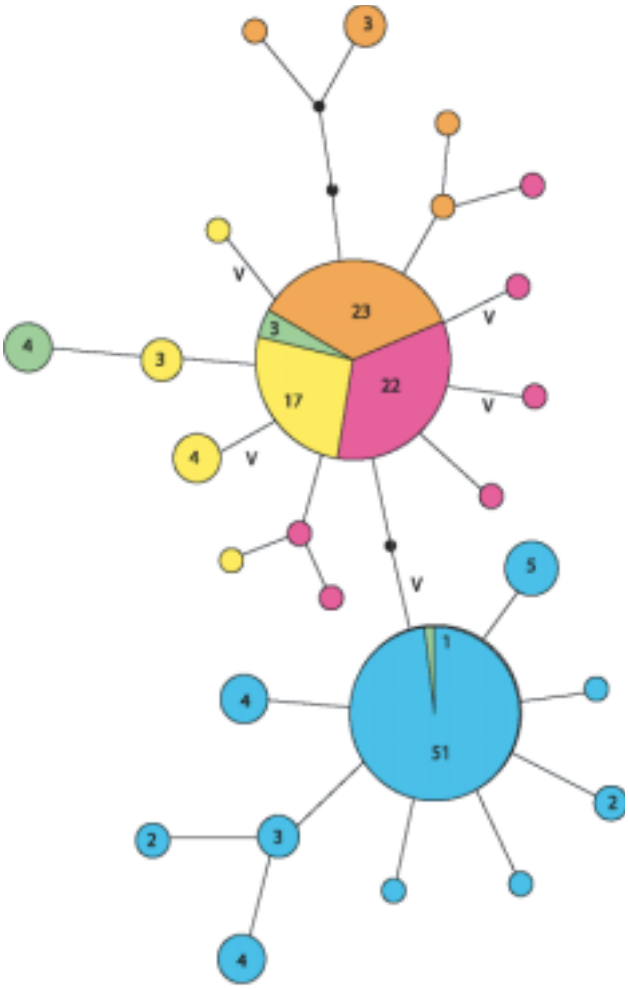

Figure 3-15. Minimum spanning network for 27 mitochondrial DNA haplotypes identified by sequencing a portion the cytochrome B gene. Colors correspond to those of the watersheds depicted in Figure 3-12. Circle area is proportional to the number of samples possessing that haplotype (the actual number is indicated if more than one. The letter "V" indicates a transversion-type mutation. Black nodes represent hypothetical haplotypes not identified in the sample.

proportional to the genetic distance between them. The basic pattern observed for this nuclear DNA analysis is similar to that observed for mitochondrial DNA: populations in the Little Kanawha watershed appear to be genetically differentiated from the four northern watersheds. In addition, there is some suggestion from the network diagram of a north-south cline.

An analysis of molecular variance can be constructed for the nuclear AFLP data, similar to the procedure for mitochondrial data. The results of the nuclear DNA analysis are strikingly different than for the mito-

chondrial DNA. Just 5% of nuclear DNA variation is due to differences between the southern watershed and the four northern watersheds. While differences among populations within each of these groups accounts for 8% of the variation, similar to mitochondrial DNA, some 87% of all nuclear DNA variation exists within populations (Figure 3-18). In contrast to the large mitochondrial F_{ST} statistic of 0.72, F_{ST} calculated for nuclear AFLPs is just 0.13. This translates into estimates of effective migration rates between populations of 0.2 based on mitochondrial DNA, and 1.7 based on nuclear DNA. Although we cannot be sure of the cause of this discrepancy, it should be noted that the estimate based on mitochondrial DNA tracks female gene flow (since mitochondria are maternally inherited) while nuclear DNA tracks both male and female gene flow.

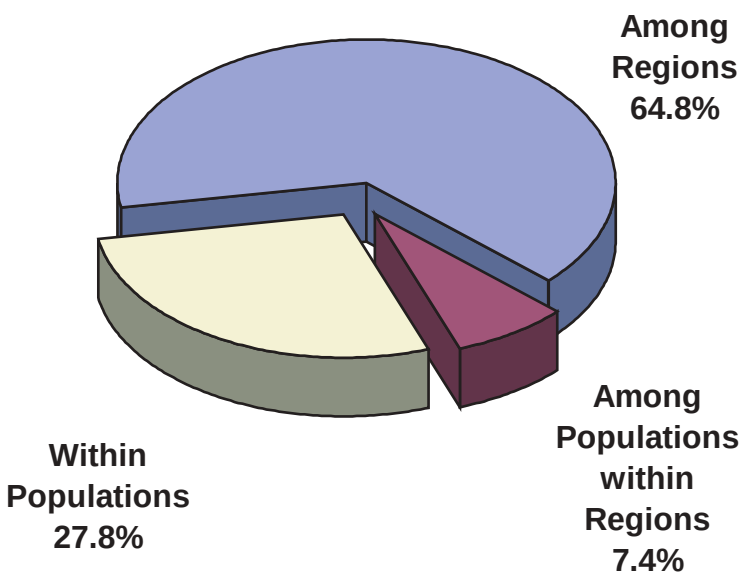

Figure 3-16. Distribution of mitochondrial DNA variation, as indicated by an analysis of molecular variance (AMOVA). For this analysis, the four northernmost watersheds were considered one region while the southernmost watershed was considered a second region. Each of the variance components was significantly greater than zero ($p < 0.0001$).

Environmental factors account for about half of the differences in mtDNA diversity within sites, and virtually all of the differences in nuclear (AFLP) diversity within sites.

Measures of genetic diversity within each population, along with several environmental measures recorded for each site, are provided in Table 3-7. AFLP diversity was actually measured as average pairwise similarity of individuals within the population (an inverse measure of genetic diversity), as was done for RAPD fingerprint data in the first case study. Mitochondrial genetic diversity was measured as the average number of mutational differences between two mitochondrial haplotypes in the population.

Six independent environmental "factors" (as determined by principal components analysis and varimax rotation) accounted for about 98% of all the environmental variance in the 25 measurements taken. Different types of measurements were related and were associated with different factors (Table 3-8). Factor 1, for example, included many geochemical measurements (aluminum, calcium, chloride, etc) while factor 4 was associated with spatial scale measurements (size of watershed, stream width and depth). The relationship between mitochondrial genetic diversity (haplotype diversity) and these six environmental factors was explored by multiple regression analysis (forward selection model). Factor 2, which was

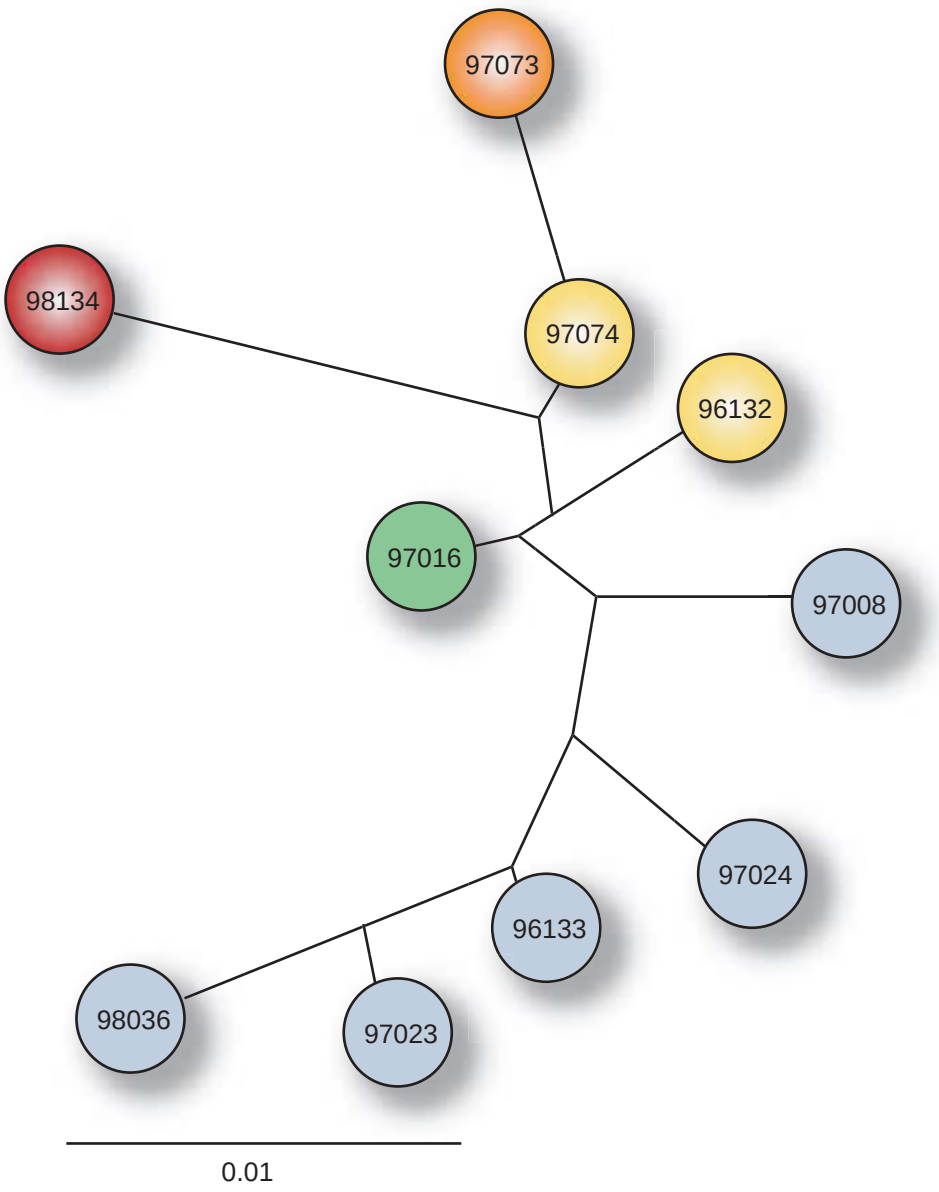

Figure 3-17. An unrooted neighbor joining network based on Nei's genetic distances (see Lynch, 1991), as estimated from AFLP polymorphisms. Populations in the same basin are colored similarly and correspond to the watersheds depicted in Figure 3-12.

associated with environmental variables nitrate, total nitrogen, total phosphorous, and organic carbon, was the only factor that was found to significantly explain mitochondrial diversity within populations. This factor explained about 50% of the differences in mitochondrial diversity ($R^2 = 0.51$, $F = 8.4$, $p = 0.02$).

Three environmental factors were found to significantly affect nuclear (AFLP) diversity within populations. Latitude and variables that were highly confounded with latitude, which included channel slope, elevation, silica and zinc concentrations, (Factor 3) explains about 43% of the differences in AFLP diversity (Table 3-9). Nitrogen, phosphorous, and organic carbon, (Factor 2) which was important in explain-

ing mitochondrial diversity, explains an additional 35% of AFLP diversity within sites. Finally, stream pH and ammonium ions (Factor 5) explain an additional 18% of AFLP diversity. Together these environmental factors explained almost all of the differences in AFLP diversity among sites ($R^2 = 0.98$). Since latitude and variables highly correlated to latitude accounted for 43% of differences, we conclude that at least 53% of differences among sites in AFLP diversity are explained by non-geographic environmental factors. Thus, it appears that both mitochondrial and nuclear genetic diversity of creek chubs in this area are responsive to stream condition.

It should be noted that we hypothesized that geochemistry would have the strongest effect on creek chub genetic diversity, since it represented the greatest environmental gradient and should be associated with mining impacts. However, no association was observed between geochemistry and genetic diversity. Interestingly, factor 2, which represented nitrogen/nitrate/phosphorus/organic carbon, was significantly associated with both mitochondrial and nuclear genetic diversity. This may suggest that agricultural inputs have a greater impact on stream condition in this area than mining inputs.

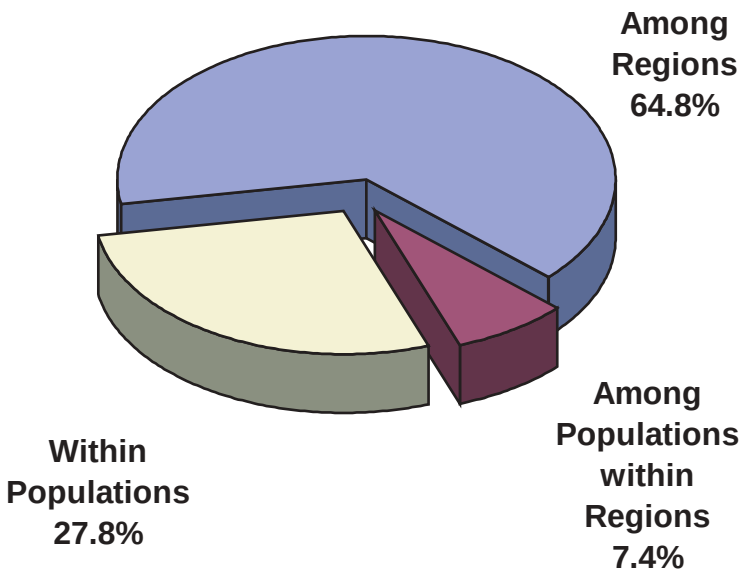

Figure 3-18. Distribution of nuclear (AFLP) DNA variation, as indicated by an Analysis of Molecular Variance (AMOVA). For this analysis, the four northernmost watersheds were considered one region while the southernmost watershed was considered a second region. Each of the variance components was significantly greater than zero ($p < 0.0001$).

3.3 Generalizations from the Case Studies

Though quite different in scope, geographic area, and in the nature of the organisms examined, the two case studies reveal several commonalities. First, the population structure of stream fishes in the Eastern United States can be quite strong, even for very common, ubiquitous species. This structure is partly a consequence of natural barriers to migration within stream networks, but the data indicates that it cannot be entirely explained by watershed boundaries. In fact, defining the USGS watersheds (8-digit hydrologic units) as the basic assessment unit would appear to be misleading for both species studied. Future ecological assessments that incorporate the population structure of stream fishes when measuring ecological condition will result in more precise estimates of the condition of fish populations.

Table 3-7. AFLP and mtDNA diversity (mean ± S.D.) and some key environmental measures for 10 sites sampled. Colors correspond to the 8-digit HUCs as depicted in Figure 3-12.

Stream ID	AFLP within population similarity	mtDNA pair wise differences	Latitude (decimal degrees)	Conductivity (µS)	Sulfate (µeq/L)	Total Nitrogen (µg/L)	Watershed Area (km^2)	pH
98-134	0.828 ± 0.025	0.507 ± 0.443	41.054	672	2849	741	28	8.31
97-073	0.899 ± 0.007	0.407 ± 0.398	40.347	1192	9222	624	57	6.61
97-074	0.882 ± 0.006	1.000 ± 0.722	40.160	3590	28569	243	49	8.31
98-132	0.879 ± 0.005	0.581 ± 0.484	39.786	308	807	273	11	8.01
97-016	0.868 ± 0.008	1.643 ± 1.078	39.606	213	447	195	98	7.77
97-008	0.845 ± 0.022	2.538 ± 1.458	39.254	339	616	2011	3	7.69
97-024	0.898 ± 0.006	1.198 ± 0.814	39.073	153	316	223	31	7.73
98-133	0.899 ± 0.005	0.548 ± 0.466	39.054	162	297	111	9	7.48
97-023	0.907 ± 0.009	0.000 ± 0.000	38.870	188	233	260	70	7.44
98-036	0.911 ± 0.005	0.308 ± 0.338	38.763	158	176	368	28	7.50

In addition to strong genetic differences between populations, both assessments also found genetic diversity within populations that varied in amount among populations. Both studies found strong associations between the amount of this genetic diversity and various measures of environmental condition. Unfortunately, the types of environmental information collected in the two studies were different so it is not possible to generalize the types of environmental factors most likely to influence levels of genetic diversity. However, the correlations that were observed should prove useful in focusing research to diagnose particular stressors that may impact fish populations. It is important to note that these inferences were made for fish populations that appeared outwardly healthy, as the species were numerically abundant at each of the sites measured. However, the genetic data revealed a scarcity of alleles in some populations, presumably a consequence of differential environmental quality, that suggest concerns about future sustainability.

Table 3-8. Environmental factors revealed by principal components analysis and the percent of total environmental variation attributed to each factor. The mean and range of values for each variable within the factors are presented.

Factor 1 *(Geochemistry) (37.43%)*	***Factor 2*** *(N/P/C) (24.33%)*	***Factor 3*** *(Latitudinal Clines) (14.14%)*	***Factor 4*** *(Spatial Scale) (11.81%)*	***Factor 5*** *(pH-Ammonium) (6.71%)*	***Factor 6*** *(Substrate Condition) (6.35%)*
Conductivity 698 µS (153-3590)	Nitrate 15 µeq/l (1-81)	Latitude 39.6º (38.76-41.05)	Watershed Area 38.4 km2 (3.2-98.1)	pH 7.69 (6.61-8.31)	Percent Substrate (> 16mm) 68% (20-96)
Aluminum 27 µg/l (5-184)	Total Nitrogen 505 µg/l (111-2,011)	Elevation 274 m (240-350)	Stream Width 5.6 m (2.4 - 11.1)	Ammonium 5.8 µeq/l (0.0- 32.8)	Embeddedness 55% (10-86)
Calcium 2,628 µeq/l (940-7,394)	Total Phosphorus 63 µg/l (5-470)	Channel Slope 1.0% (0.10-2.10)	Stream Depth 29.9 cm (13.2 - 59.4)		Percent Riffle 31% (5-50)
Chloride 1,044 µeq/l (85-6,471)	Organic Carbon 80 mg/l (39-179)	Silica 6 mg/l (3-13)			
Potassium 80 µeq/l (39-179)		Zinc 0.01 mg/l (0.00-0.04)			
Magnesium 1,366 µeq/l (341-6,066)					
Sodium 3,669 µeq/l (192-27,665)					
Sulfate 4,353 µeq/l (176-28,569)					

Table 3-9. Forward stepwise multiple regression of AFLP within population similarity value as dependent variable. Three variables met the 0.05 significance level for entry into the model.

Environmental Factor	***partial*** R^2	***model*** R^2	***F value***	***Pr > F***
PCA Factor 3 (Latitudinal clines)	0.4328	0.4328	6.10	0.0387
PCA Factor 2 (N/P/C)	0.3489	0.7917	12.06	0.0104
PCA Factor 5 (pH/Ammonium)	0.1841	0.9758	45.60	0.0005

4 Considerations when Implementing a Genetic Diversity Assessment

Suggestions that genetic diversity should be used as an indicator of ecological health are not new (e.g., Beardmore *et al.*, 1980; Nevo *et al.*, 1988; Foré *et al.*, 1995a, 1995b). However, specific guidance on how genetic markers should be incorporated into an ecological assessment program is lacking. The overwhelming majority of reported scientific studies of genetic diversity are at relatively small scales, incorporating assays of at most a few dozen populations. Aspects of how to scale-up to region-wide analyses with dense geographic sampling, including data management and quality assurance issues, have not been considered. In addition, the focus of genetic diversity studies in the scientific literature is usually on the status of the species under study, not the ecosystem. The US EPA is currently engaged in several studies designed to evaluate the utility and practicality of implementing genetic diversity indicators as part of ecological assessment or monitoring. Based on our experiences with these studies and relevant information from the scientific literature, several guidelines are suggested.

4.1 Sampling Design

Two different types of sampling designs have traditionally been employed for ecological assessments: source-biased studies, in which sites with known exposures are compared to reference sites, and region-wide studies, in which a large number of sites are sampled according to a defined sampling scheme in order to create a regional profile. Some studies may have aspects of both designs, in which sites with known exposures are compared to a relatively large number of "reference" sites within the region. Both designs are amenable to genetic diversity analysis. Many examples of the source-biased design applied to genetic diversity exist in the ecotoxicological literature (see Table 2-1). In addition, there are many examples in the conservation genetics literature of genetic diversity analyses that incorporate regional scales, although they rarely include large numbers of sample sites within the region. To our knowledge, no examples yet exist of intensive regional ecological assessments that have utilized a genetic diversity indicator.

The source-biased design has obvious cost advantages when the assessment question of interest is whether a known, local exposure has an impact on the genetic diversity of resident populations. However, considerable care must be exercised when implementing this design. Because the intent of this design is to measure a recent genetic change, the reference population(s) will ideally be identical to the test population(s) in all aspects except for the application of the specific exposure, yet independent of the test population(s) following the exposure. Thus, the populations must have had similar genetic diversity before the exposure, either because they recently diverged or because they experienced high gene flow prior to the exposure. In addition, significant gene flow between the populations must have stopped immediately following the exposure and any genetic differentiation that occurred must have been due to the exposure and not to other population or environment-related factors. These standards are likely to be difficult to meet. In practice, genetic diversity is often measured at a number of reference sites and compared to the exposed site. If genetic diversity at the exposed site is outside the norm for the reference sites then the exposure is implicated as the cause of the change in genetic diversity. Here too, there can be difficulties with interpretation. Often, reference populations are chosen to be geographically distant from the exposed population in order to ensure that they represent "reference conditions". Typically, it is not clear that the reference populations are not each more closely related to each other than any is to the exposed population and that any genetic diversity differences uncovered did not predate the exposure.

Regional studies offer much greater ability to characterize patterns of intraspecific genetic diversity and their possible causes than do source-biased studies. Genetic diversity will naturally vary among popula-

tions for a variety of reasons, including variation in the size of populations that different habitats can support, as well as evolutionary relationships and patterns of dispersal among populations. These natural levels of genetic diversity are indicative of fundamental population data, such as effective population sizes and population boundaries (section 2.1). In addition, any attempt to determine whether anthropogenic factors have influenced present levels of genetic diversity must be able to distinguish historical (evolutionary) patterns from recent change. Since regional assessments allow better characterization of the natural variation in genetic diversity measures, they can provide guidance for selecting specific areas for more intensive study. For example, if genetic diversity of one population is determined to be low, it can be compared to evolutionarily similar populations to determine whether a recent genetic change is implicated.

The natural synergisms that genetic diversity data lend to landscape-level analyses and species assemblage studies suggest that incorporation of genetic diversity sampling into existing regional assessment programs, including EMAP and Regional EMAP programs, is the preferred strategy to obtain genetic diversity data at present.

The natural synergisms that genetic diversity data lend to landscape-level analyses and species assemblage studies suggest that incorporation of genetic diversity sampling into existing regional assessment programs, including EMAP and Regional EMAP programs, is the preferred strategy to obtain genetic diversity data at present. Sampling of target species for genetic diversity analyses can easily be accomplished within existing EMAP guidelines, and will help reduce costs of sample collection. One of the advantages of DNA-based analyses is that sufficient DNA can be obtained from a single fish fin-clip to perform a large number of genetic analyses. Thus, tissue can easily be collected in the course of normal field identification procedures and, in most cases, specimens can be released back into the environment.

In the long run, the most efficient method to measure anthropogenic changes in genetic diversity is to measure genetic changes directly through temporal monitoring. For this purpose, regional assessments provide an excellent means to collect the necessary baseline genetic data for future comparisons. In addition to regional-scale assessments, intensive temporal analyses of genetic changes at a network of index sites will be extremely valuable. Index sites typically are assessed with a greater range of diagnostic and condition indicators, which will allow for better integration of the genetic data. Since index sites are intended for long-term monitoring, they provide an excellent opportunity to measure the temporal scale of genetic changes. For both regional and index site studies, it will be critical that DNA or tissue is archived for future analyses as part of the assessment. Given the rapid changes occurring in molecular methodologies, it is likely that whatever marker is used to measure genetic diversity initially will not be the optimal strategy at later stages of the assessment. The availability of archived samples will allow future retrospective analyses to assess genetic changes using the most appropriate technologies available.

In the long run, the most efficient method to measure anthropogenic changes in genetic diversity is to measure genetic changes directly through temporal monitoring.

Scale-up issues. Several project management issues emerge when the scale of genetic analysis changes from assessment of genetic structure at a few sites at a single point in time to assessment at regional scales and/or over time periods that may encompass decades. Clearly, the greater management complexity and cost of this type of project requires greater forethought in design of the genetic survey. The utility of a pilot study to guide project management cannot be overemphasized. The pilot study serves to validate the choice of molecular markers and species in the study region and allows for initial assessment of the feasibility of more intensive or large-scale sampling (Baverstock and Moritz, 1996). It is very likely that the project plan will be redefined based on the results of the pilot study. For example, a target species may be difficult to sample within the region or a number of molecular markers that were found to be useful in other areas may lack polymorphism (and thus discriminatory power) within the region. A pilot study also may determine whether the scale or intensity of sampling for a regional assessment is appropriate.

Management of a large genetic diversity survey will be logistically simpler if it can be divided into smaller units that are analyzed individually. For example, the EPA's pilot study of genetic diversity for central stonerollers throughout the Eastern Cornbelt Plains Ecoregion (section 3.1) was divided into analysis units that were equivalent to large watersheds or basins. In addition, analyses were completed for central stonerollers before attempting to assess genetic diversity in additional species. Geographical and species-stratified analysis has the additional advantage that fairly intensive genetic diversity assessments can be completed for specific basins on a regular basis throughout the life of the project. While the advantages of such compartmentalization of analyses are clear, it does lead to a potential for bias if variation in laboratory procedures occurs over time (thus, between sample units). Inclusion of duplicate samples from prior analyses as a type of positive control against temporal variation in laboratory procedures should control for this type of bias.

Additional scale-up issues involve planning for adequate data management structure and are dealt with in section 4.6.

4.2 Species Choice

Most genetic diversity studies in the scientific literature are focused on conservation or management of a particular species, usually one that is threatened, endangered, or of some economic importance. In choosing species as indicators of environmental health, other issues clearly come into play. Table 4-1 presents "optimal" species attributes for environmental monitoring. Many of these species attributes stem from consideration of basic population genetics. Genetic diversity of species that are highly sensitive to degraded habitat and that have short generation intervals will respond more quickly and more dramatically than other species. Species with low dispersal ability will have higher exposure to specific environments and may provide finer resolution of environmental differences between sites. Asexual species, including microbes and algae, are not optimal mainly because the distinction between intraspecific and interspecific genetic diversity is blurred. Valid use can be made of such species (e.g., Ford *et al.*, 1998) but they become functionally equivalent to species assemblage indicators.

Other species characteristics listed in Table 4-1 help make collection, analysis, and interpretation of the data simpler. Selection of species that are easily distinguished morphologically will ensure that genetic diversity is measured at the intraspecific level, and that comparisons are not erroneously made between different species (in fact, cryptic species complexes are readily diagnosed using molecular markers, which is one advantage of combining analysis of genetic diversity with species assemblage assessments). Selection of broadly distributed species allows simpler analysis of scale issues. Use of species that are important to resource managers will allow easier integration of genetic diversity monitoring into existing monitoring programs. Species that are moderately abundant within the study area are easier to

Table 4-1. Optimal characteristics of species assessed with a genetic diversity indicator.

Optimal Species Characteristics
• Short generation interval • Moderate-high exposure to stressor(s) • Moderate-high sensitivity to stressor(s) • Low-moderate dispersal ability or highly philopatric • Sexual reproduction • Native species • Broad distribution • Moderate abundance • Management importance • Morphologically distinct • Known life-history parameters (age structure, sex ratio, etc) • Availability of comparative genetic and demographic data • Availability of historical DNA or tissue samples • Amenable to laboratory culture

collect, although these species are usually not management priorities. The population genetic structure of species that have not been excessively moved around is generally easier to interpret than the structure of introduced species. This is because the stocking history of introduced species is generally poorly known; non-native species could be useful indicators in cases where the history of introductions and their sources are well documented. Availability of historical data, including the availability of archival samples (DNA, fins, scales, or whole preserved specimens) is useful for reconstructing changes in genetic diversity that may have occurred prior to or during known exposures in the past. Finally, the selection of species that can be cultured in the laboratory will aid in assigning causality to genetic changes, if such studies are desired in the future.

Ideally, the indicator will be applied to several species, since a multi-species index should better predict ecosystem status than a single-species index. Genetic responses of individual species are not always predictable (Gillespie and Guttman, 1999), as different species "see" different aspects of the environment, and not always what we expect. If several species are selected then additional considerations become relevant, such as sampling from phylogenetically and ecologically diverse taxa.

4.3 Which Genetic Marker?

Several books have been published in recent years that review the biological and analytical properties of different molecular markers (e.g., Avise, 1994; Hillis *et al.*, 1996; Caetano-Anolles and Gresshoff, 1997). A general consensus is that no genetic marker is best for all applications and in the hands of all investigators; each provides different insights and requires different levels of investment in equipment and training. Here, we will summarize the properties of some of the most common genetic markers and discuss their relative strengths and weaknesses as ecological indicators.

Allozymes. Allozyme electrophoresis is a simple and time-honored technique in the field of population genetics. The principle of the technique is that allelic forms of enzyme proteins with different net charges will have different mobilities when induced to move through a matrix by application of an electric current (electrophoresis). Alternative forms of the enzyme at the enzyme locus (alleles) migrate different distances through the matrix and are visualized by histochemical staining (Figure 4-1). Numerous manuals have been developed that outline general equipments needs, procedures, and gel pattern (zymogram) interpretations (see May, 1992; Murphy *et al.*, 1996; and references therein).

Although allozymes are often thought of as "old technology", they have some clear advantages over other genetic markers as ecological indicators. Few other markers can match allozymes in the simplicity and economy of standard procedures. Allozymes have a much longer history than other genetic markers and have been analyzed in many more laboratories so the historical database of comparative population genetic data is much larger for this marker than for any other. In fact, it is highly likely that allozyme datasets can be found for any of the common stream fishes in the country. In comparison to some other markers, allozyme techniques suffer from a limited number of marker loci available for study and a limited number of alleles per locus. Less than a dozen polymorphic markers are assessed in typical studies, and most of these markers only segregate for two or three variant alleles. As noted earlier, allozyme loci are more likely to be affected by natural selection than most DNA markers, which may bias estimates of gene flow and genetic diversity. For example, allozyme markers suggested significant gene flow in oysters along the eastern and Gulf coasts of North America, but both mitochondrial and nuclear DNA markers indicated a sharp biogeographic boundary between northern and southern populations in northeastern Florida (reviewed in Avise, 1994).

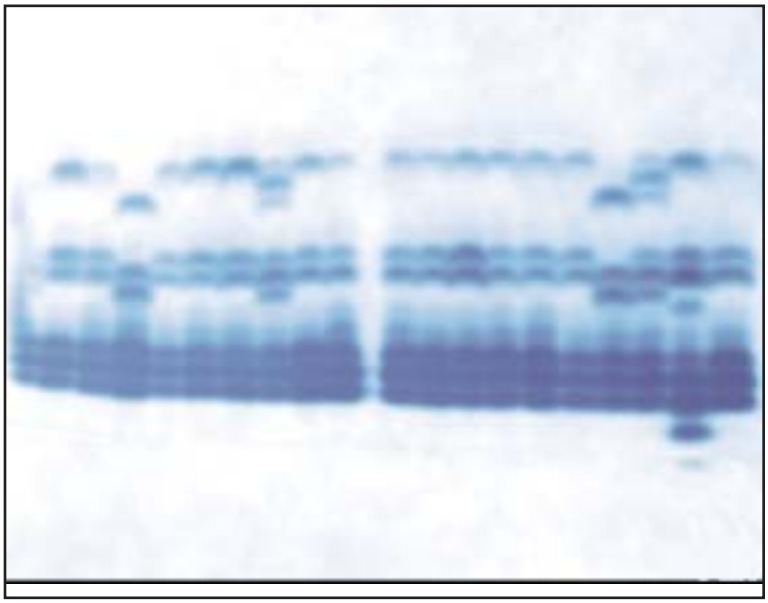

Figure 4-1. A histochemically stained starch gel showing GPI allozyme loci of rainbow trout. Each vertical lane represents a different individual.

Mitochondrial DNA Sequencing, RFLPs, and Prescreening Strategies.

Mitochondrial DNA is a circular DNA molecule containing about 16,500 base pairs that is present in multiple copies in the cells of eukaryotic organisms. Mitochondrial DNA has a number of properties that lend it to ecological assessments. Mutation rates tend to be higher than for most nuclear DNA regions, so large numbers of alleles (called haplotypes) are generated. Each individual usually only possesses one mitochondrial haplotype, which it typically inherits from its maternal parent. In addition, recombination within the mitochondrial genome appears to be rare or absent. Unlike different allozyme alleles, mitochondrial DNA sequences (Figure 4-2) can be analyzed to determine patterns of evolutionary relationships between different haplotypes. Thus, analysis of mitochondrial DNA sequences adds a different dimension to the analysis of genetic diversity; one can move beyond asking whether two individuals are the same or different at a genetic locus to ask *how* different they are at that locus. This provides a straightforward method for assessing (maternal) genealogical relationships between individuals of a population, and between individuals of different populations and different species.

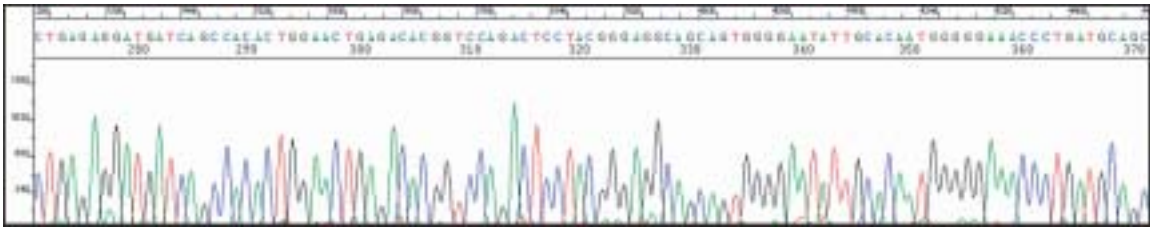

Figure 4-2. Electropherogram of DNA sequence generated by an automated genetic analyzer. Identification of mutational relationships among mitochondrial types (haplotypes) can elucidate patterns of evolutionary relationships among populations.

Mitochondrial DNA is not without drawbacks, the most important of which is that the lack of recombination within the molecule causes the entire mitochondrial genome to behave effectively as a single genetic marker; different mitochondrial genes are linked and therefore not independent. Since the history of a mitochondrial lineage is not identical to the population history (most notably, it says nothing about male contributions), interpretations made strictly from mitochondrial DNA may be erroneous.

The tremendous information content derived from DNA sequencing comes at a cost in terms of equipment, supplies, and technical expertise (Figure 4-3). Constant technological improvements are leading to rapid reductions in these costs. For example, the development of the polymerase chain reaction (PCR) and "universal" PCR primers has negated the need to isolate mitochondrial DNA away from nuclear DNA, or to clone specific fragments prior to sequencing (instead, the target sequence is simply PCR amplified). Meanwhile, a number of techniques have been developed that reduce the need for DNA sequencing or the number of required sequencing reactions. A common strategy is to screen for restriction fragment length polymorphisms (RFLPs). RFLPs provide a coarse indicator of DNA sequence variability, typically capturing less than one-eighth of the DNA sequence variability in a region. However, RFLP patterns contain information about relationships between haplotypes and can be analyzed relatively inexpensively. Other methods are used to "prescreen" mitochondrial DNA, so that only unique or previously uncharacterized haplotypes are sequenced. These include single strand conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and various commercial strategies. These techniques typically identify 80% to 100% of single-base mutations within DNA, but usually say little about haplotype relationships. Still, when combined with DNA sequencing, they can be more informative than RFLP analysis with only slightly more technical difficulty and cost (Figure 4-3).

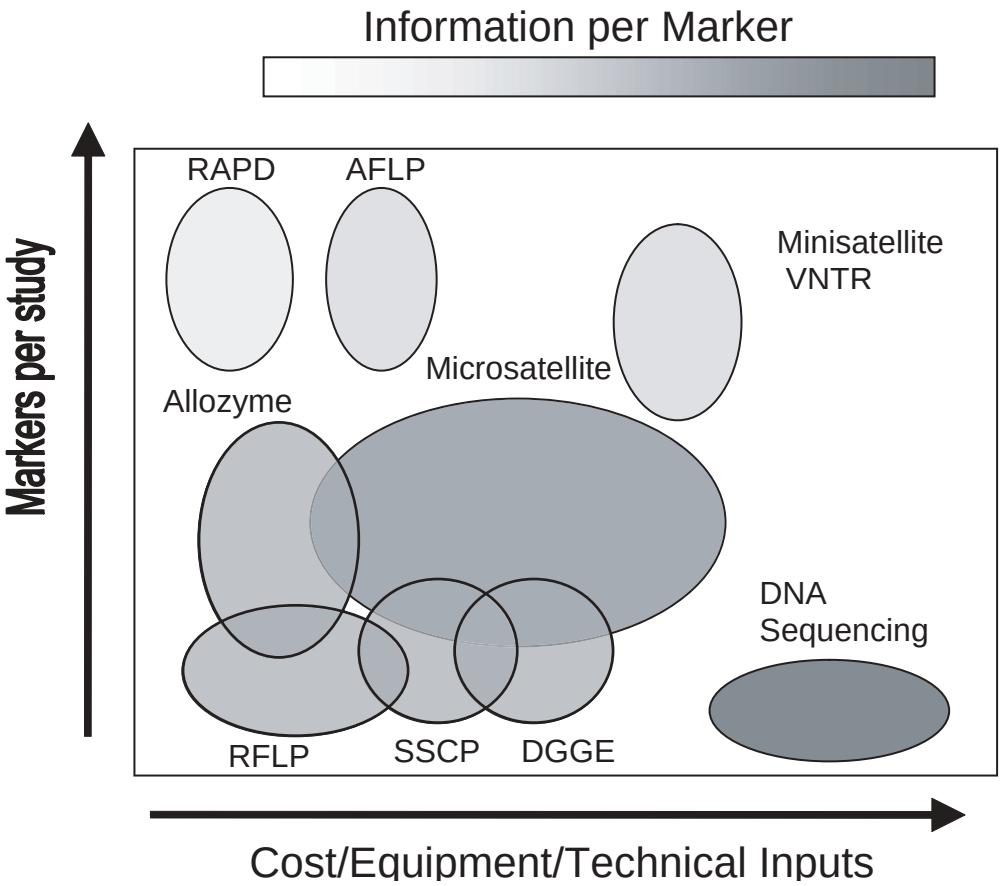

Figure 4-3. Relative advantages and disadvantages of different molecular marker strategies in relation to information per marker (darker colors in the figure being more informative), the number of markers per typical study, and costs per study in terms of capital outlay and technical expertise. The figure is not based on quantitative data and is presented for illustration purposes only.

Nuclear DNA Sequencing, RFLPs, and Prescreening Strategies. Strategies that are available for analysis of mitochondrial DNA also are available for the analysis of nuclear DNA genes, with similar advantages and limitations. The general strategy is often referred to as single-copy nuclear DNA (scnDNA) analysis. Typically, non-protein coding intervening sequences (introns) or flanking regions are targeted for analysis since they are usually more polymorphic than coding sequences. The primary advantage of scnDNA analysis is that many more genetic markers that are independent are available for analysis, so it can be highly complementary to mitochondrial DNA analysis. The development of gene sequence databases for many organisms, combined with PCR technology, has made available a number of well-studied genes for population analyses. A number of "universal" PCR primers have been published (e.g., Palumbi, 1996) to aid in the development of genetic markers for different species, although a certain amount of primer modification is often required. Because many of the gene sequences available for analysis are believed to have an impact on fitness, these, like allozymes, have the potential to be developed as diagnostic indicators of natural selection (and thus population stress). There are a number of disadvantages with scnDNA markers. In general, levels of polymorphism are low compared to mitochondrial DNA and some other nuclear DNA markers. In addition, the analysis is relatively intensive, even when using mutation-prescreening techniques, so relatively few scnDNA markers are generally analyzed per study.

Multi-locus DNA Fingerprints. DNA fingerprinting is a strategy that is in many respects the opposite of scnDNA analysis. Instead of targeting single, relatively well-characterized genes, DNA fingerprints target many anonymous chromosomal regions for analysis simultaneously. Typically, little is known about these regions except that they possess a small region of similarity to specific probes or PCR primers. Any region that has such homology presents as a distinct DNA fragment or "band" following gel electrophoresis. For any one individual, the pattern of presence and absence of bands can be highly complex, resembling a bar code. With the most variable markers, individuals can be uniquely identified by these band patterns. The most common DNA fingerprinting technique is random amplified polymorphic DNA (RAPD) analysis (Figure 4-4a), in which short primers of arbitrary sequence are used to amplify DNA fragments from 10-50 discrete regions in the genome. A newer method called amplified fragment length polymorphism (AFLP) analysis (Figure 4-4b) is similar, but depends on amplification of polymorphic fragments generated by restriction enzymes (RFLP) from arbitrary regions of the genome.

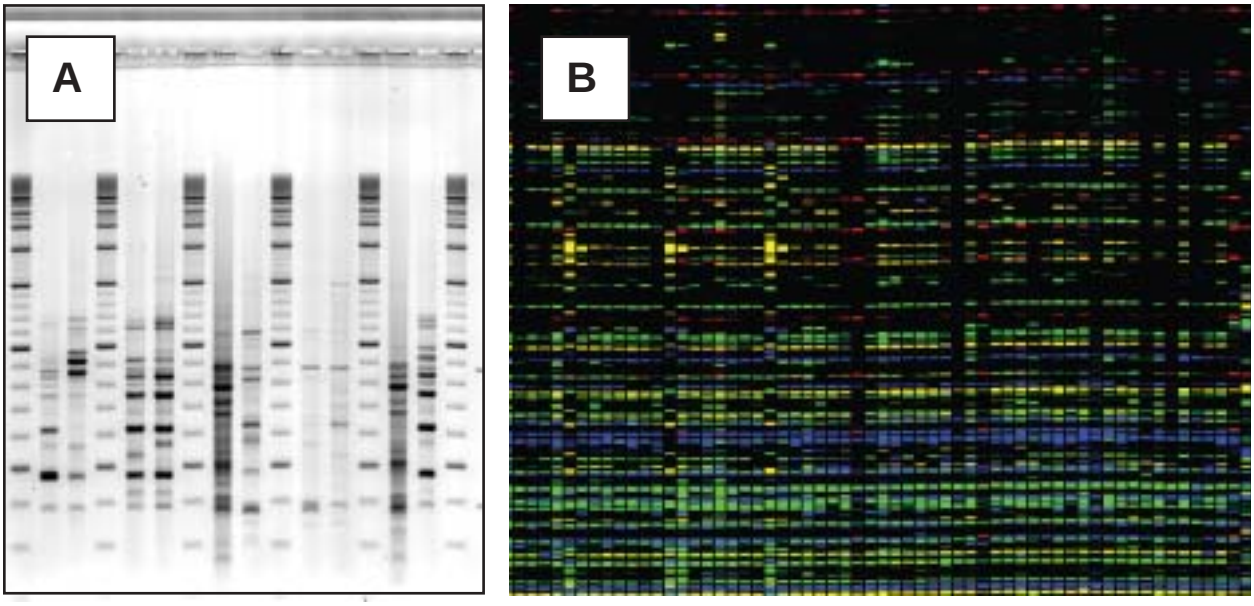

Figure 4-4. Examples of multilocus DNA fingerprints. Both RAPD (A) and AFLP (B) fragments can be generated without a priori knowledge of an organism's sequence. As dominant markers, homozygous and heterozygous individuals cannot be distinguished. The AFLP fingerprint here differs from Figure 3-14 because this is a multiplex AFLP reaction generated with an automated genetic analyzer.

There are two principal advantages of multilocus fingerprints for ecological assessments. The first is that no sequence information about the genome of the organism is needed in order to apply the methods. Thus, marker development costs are minimal and species can be chosen for analysis based on ecological or management criteria rather than the amount of sequence information already known. The second advantage is that genetic differences between individuals can be distinguished with relative economy. Dozens to hundreds of markers are analyzed in a typical DNA fingerprinting study.

The overriding disadvantage of DNA fingerprints is the poor quality of genetic information from each individual fingerprint band. Individual fingerprint bands cannot reliably be assigned to independent genetic loci. More importantly, RAPD and AFLP markers are dominant markers, which means that individuals that are heterozygous for a marker (i.e, only one chromosome of the pair has the marker) cannot be distinguished from individuals that are homozygous for the marker (both chromosomes have the marker), significantly decreasing the genetic information available. As a result of these ambiguities, comparisons are generally made in terms of overall 'similarities', taking into account the proportion of bands that are shared between individuals. Another concern with DNA fingerprinting methods is that sophisticated procedures must be implemented to reliably sort through the complex fingerprint patterns to identify homologous fingerprint bands from different individuals. Related to this is concern about the overall reliability of fingerprinting methods, particularly RAPD fingerprints. The RAPD technique is known to be very fickle, and adherence to exact protocols by different labs, often including use of the same brand of equipment and reagents, is considered critical to repeatability.

Microsatellite DNA Markers. Microsatellite DNA, also called simple sequence repeats (SSRs) are regions of repetitive DNA that consist of tandem repeats of a core sequence of two to five base pairs, such as (CA), (TAGA), and (CAT). Different alleles at a microsatellite locus differ in the number of tandem repeats of the core sequence. These sequences appear to be ubiquitous in the genomes of eukaryotes, and thousands of potential microsatellite markers could be developed for most species.

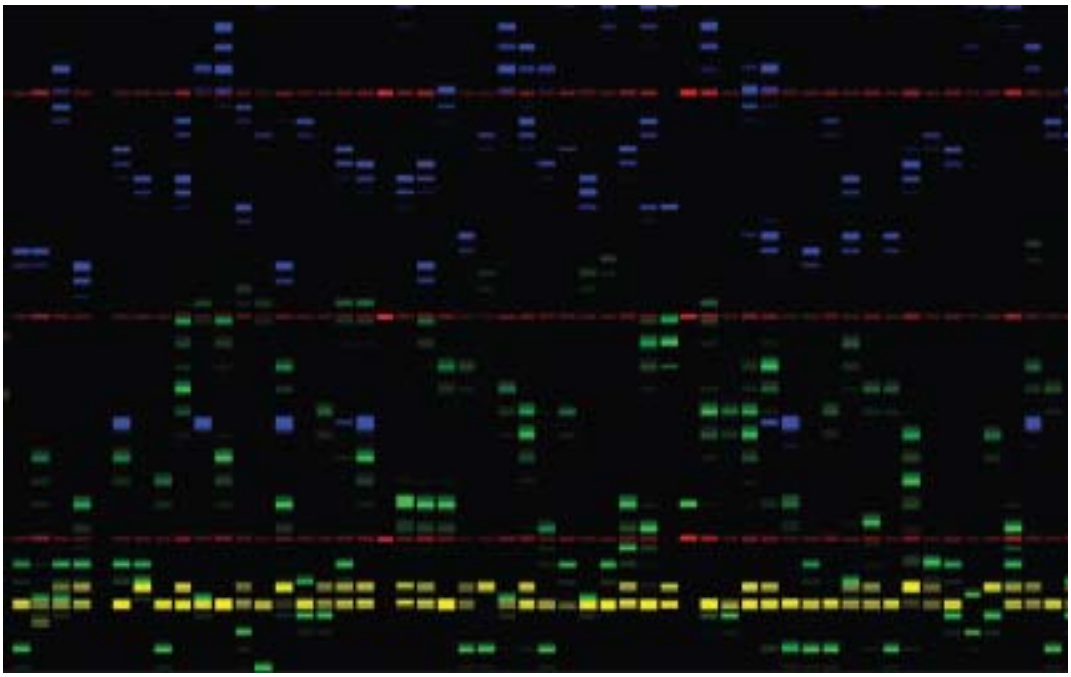

Figure 4-5. Flourescently labeled microsatellites detected using an automated genetic analyzer. Use of flourescently labeled markers allows differentiation between multiple loci (illustrated above by blue, green and yellow labeled markers) within the same reaction (multiplex PCR) thus reducing cost and increasing throughput.

Microsatellite DNA markers have some tremendous strengths for ecological assessments. They are subject to very high mutation rates relative to scnDNA, sometimes producing dozens of alleles (Figure 4-5). Like allozymes and scnDNA markers, inheritance of microsatellite markers is codominant, so heterozygotes can usually be reliably differentiated from homozygotes. The proportion of individuals that are heterozygous in a population is much higher than for other nuclear loci, often approaching 100%. Although heterozygosity at microsatellite loci is somewhat less sensitive to genetic bottlenecks than mitochondrial DNA diversity, it is much more sensitive than other nuclear DNA markers due to the large number of rare segregating alleles at these loci. Loss of these rare alleles actually provides a more sensitive measure of population bottlenecks than does heterozygosity (Leberg, 1992). It appears that the predominant mode of mutation is to an allele one repeat-unit different from the original allele [e.g., from $(CA)^{12}$ to $(CA)^{13}$], thus genealogical information can be captured from allelic relationships of microsatellite loci, although this information is less precise than that captured from DNA sequence analysis of mitochondrial DNA and nuclear genes. The primary disadvantage of microsatellite DNA markers is development cost. Technical expertise necessary for microsatellite marker development is greater than for any of the other markers listed, although, like DNA sequencing, the technical demands are decreasing. Microsatellite markers are developed from non-protein coding DNA regions and, therefore are not conserved across taxa, so that microsatellite DNA markers developed for one species are often only useful for very similar species. The number of organisms for which microsatellite markers have been developed is increasing rapidly, so it is possible that microsatellite development will be less costly in the near future. In addition, the very large number of alleles present at some microsatellite loci requires large sample sizes be used to estimate allele frequencies accurately.

Recommendations. This report has considered only a subset of the available genetic markers, but these are the most common and well supported in the scientific literature. The "best" marker for ecological assessments will vary, depending on the specifics of each situation. Until reliable methods are developed that allow economical analysis of nuclear DNA sequences from more than just a few genes per study, RFLP, SSCP, DGGE and sequence analysis of nuclear genes will likely be less informative than allozyme or microsatellite studies per unit effort. Mitochondrial DNA can be highly informative and offers insights not available from analysis of nuclear DNA. However, information from mitochondrial DNA may be misleading if interpreted alone so it is recommended that mitochondrial DNA be assessed only in conjunction with other markers.

Among the remaining markers, microsatellites undoubtedly offer the best combination of information per genetic marker and potential for analysis of many genetic markers. Although most microsatellite studies of natural populations to date have utilized relatively few microsatellite markers, there is now no technical reason why dozens, even hundreds of microsatellite markers cannot be developed and applied to genetic analyses. The technical challenges of microsatellite development can be overcome (for example, several commercial laboratories will now develop microsatellite markers on contract), however, an advanced laboratory is still required for microsatellite analysis. Reliable scoring of microsatellite markers requires the use of labeled PCR primers (either isotope or fluorescence) for visualization so, minimally, laboratories must have the ability to detect these labels. For large-scale, regional analyses utilizing many microsatellite markers, automated laboratory analysis using commercial genetic analyzers (automated sequencers) is essential.

The many advantages of allozymes, (economy, standardized methods, large existing database of information, homology of loci across species) should not be overlooked, particularly when microsatellite analysis is infeasible. Targeted analysis of specific allozyme loci (e.g., GPI) also may be useful as diagnostic indicators of specific stressors (e.g., heavy metals), and could complement analyses of other genetic markers. However, the requirements for ultra-cold storage of tissue samples in the lab and in

the field may make allozyme analysis impractical for regional analyses, particularly if the genetic collections are done as part of a multi-indicator assessment. In addition, if genetic diversity of allozymes is too low overall in the indicator species (as determined, perhaps, by a small pilot study), or if lethal sampling is not acceptable, then other methods should be explored. AFLPs and RAPDs, in that order, should be considered if neither allozyme nor microsatellite studies are feasible. Both methods allow assessment of many different genetic markers, but identification of AFLP markers is believed to be more repeatable between laboratories. However, AFLP analysis, like microsatellites, requires a more advanced laboratory able to detect isotopically or fluorescently labeled PCR products. Mitochondrial DNA analysis may be combined with any of the above nuclear markers to provide semi-independent genetic information. For example, analysis of evolutionary relationships among mitochondrial haplotypes may provide information on the historical biogeography of the species. Such information can help interpret patterns of genetic diversity in nuclear markers. A flow chart to aid in choosing genetic markers is provided in Figure 4-6.

4.4 Sample Size Considerations

Clearly, statistical power to detect differences in genetic diversity within populations and to detect genetic differentiation among populations will increase with increasing sample size. Two different sample sizes need to be considered: the number of individuals sampled per population and the number of markers assessed per individual. For any one marker, the ability to discriminate allele frequency differences between populations is low so large numbers of individuals need to be sampled from each population. In general, as more markers are assessed per individual, fewer individuals need to be sampled from each population. However, the balance between the number of markers and the number of individuals varies with the marker chosen. Recommendations for determining appropriate sample sizes are reviewed in current literature (Baverstock and Moritz, 1996).

In general, genetic markers that allow estimation of molecular relationships (DNA sequences, RFLP, and microsatellites) should require smaller sample sizes to achieve the same statistical power as markers that only consider allele frequencies, although this depends on the level and complexity of molecular relationships. Estimates of allele frequencies for microsatellite markers with high polymorphism (e.g., 10 or more alleles) can have very high standard errors since few individuals in the sample will possess any one allele.

For dominant markers (RAPD, AFLP) in which heterozygotes cannot be distinguished from one of the homozygotes, less information is available per locus and therefore more individuals or more markers must be sampled for the same statistical power as analysis of codominant loci. Often, logistical problems limit the number of individuals that can be sampled so statistical power is gained by sampling as many loci as possible. Simulation studies by Mariette *et al*. (1999) suggest that at least four times as many AFLP markers are needed as microsatellite markers to estimate genetic diversity within populations that are at equilibrium between migration and genetic drift. Comparatively more AFLP or RAPD markers will be needed to measure genetic diversity of populations that are not at equilibrium. Mitochondrial DNA, which is only transmitted from female parent to offspring (haploid inheritance), also has less genetic information than a single codominant marker, based on analysis of haplotype frequencies alone. Reduction in the standard error of estimates of haplotype frequencies can only be accomplished by increasing the number of individuals sampled.

Although it is difficult to come up with specific numbers, as a rule of thumb, sample sizes of between 50 and 100 samples per site are typically targeted in population genetics studies, although actual sample sizes are often lower. Analyses that utilize dominant marker systems should aim to assess between 100-

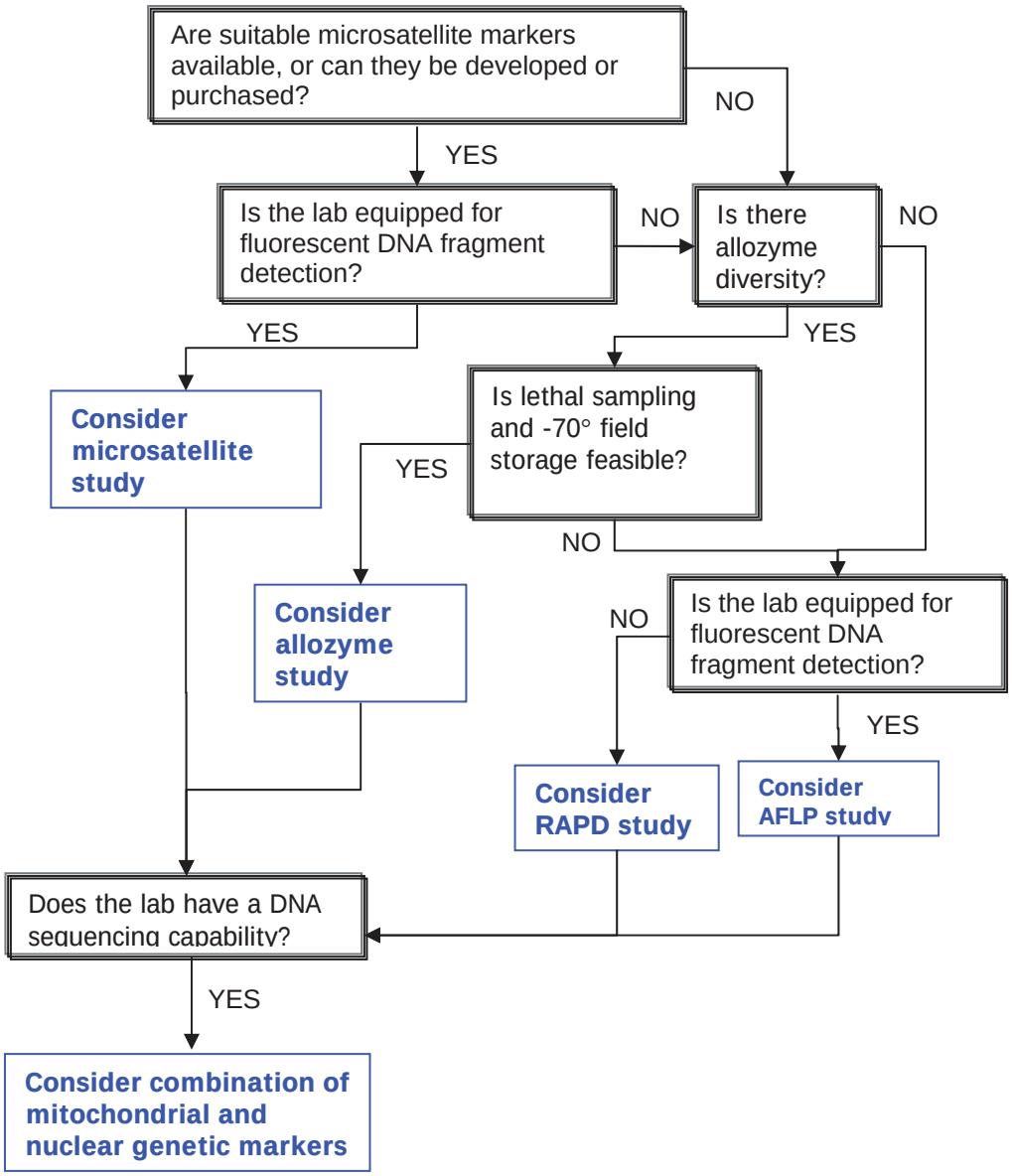

Figure 4-6. Flow diagram for deciding the best genetic marker or combination of genetic markers in relation to available resources.

200 polymorphic markers. Analyses that utilize codominant markers should aim for between 20 and 50 polymorphic loci.

4.5 Personnel Training and Specialized Equipment

Significant technical expertise is required for field sampling, laboratory analysis, and data analysis and interpretation. The degree of field expertise is equivalent to that needed for species assemblage indicators. Personnel must be able to operate sampling gear such as backpack electroshockers or seines effectively. In addition, they must have the ability to identify target species in the field. In practice, species discriminatory skills are probably less important than for assemblage indicators because the validity of the field identification will likely be confirmed by the genetic analysis.

Laboratory analyses require varying levels of skills. DNA extraction and quantitation techniques have become highly commercialized and many simple kits can be used that yield DNA of suitable quality and concentration for PCR-based analyses. The primary skill necessary is accurate pipetting. PCR analysis also is relatively straightforward and usually only requires good pipetting skills. However, a number of factors can disrupt the PCR process, and troubleshooting problems is a common occurrence in PCR assays. For this reason it is recommended that M.S.-level person with molecular biology training be on hand to supervise or consult during the PCR phase. A similar skill-level is recommended for gel preparation, sample loading and electrophoresis, and for operating automated DNA sequencers of genetic analyzers. Interpretation of gel patterns to determine the allelic complement at each marker analyzed generally requires a skilled, M.S.-level molecular biologist. In general, improvements in technology, such as highly robotic capillary electrophoresis systems for genetic analysis, have decreased the training requirements necessary to perform genetic analyses. Development of microsatellite markers requires sophisticated molecular biology skills.

Equipment requirements to perform different laboratory operations are described in Table 4-2. A number of software packages are available for genetic analysis, and similar analyses can be performed with general-purpose statistical software such as SAS©. Selection and interpretation of appropriate indices is best done in consultation with a population geneticist or a statistician who is familiar with genetic data.

4.6 Information Management

A typical genetic diversity study will generate large amounts of data. Critical data sets include the locations and dates of sampling, the number of individuals of each species that are sampled per site, and the genotype of each individual at each of the molecular markers analyzed. These data sets must be related to other databases that may exist, including phenotypic data (age, size, developmental abnormalities) biomarker data, chemical and physical habitat data, assemblage indicator data, and landscape-level data. This suggests that use of relational database software will be useful to manage the data, particularly if this software has already been incorporated to manage linked databases. However, simpler database management tools, including spreadsheets and simple database software, can be appropriate for less complex datasets.

Large numbers of tissue samples and DNA extractions will be collected that must be archived for validation purposes and to aid future retrospective assessments. Minimally, a database is needed that documents for each sample a unique sample ID, a population ID, the storage location of the tissue sample, the storage location of the DNA sample, dates of collection, DNA extraction, DNA quantification, as well as amounts and quality assessment of the archived material. If voucher specimens were collected along with the genetic samples then the database should include this link as well. Field data collection

forms and forms that document dates of laboratory manipulations and spatial orientations of samples during those manipulations (DNA extraction, PCR, electrophoresis, marker scoring) will need to be physically archived.

Metadata requirements include documentation of field collection procedures, as well as detailed laboratory methods (see appendices) and data analysis procedures. The procedure for translating genotypic data for an array of genetic markers into a data file must be explained, including descriptors of each of the fields in the data file. Similarly, documentation of the rationale and procedures for the statistical analyses, including software documentation, are needed.

4.7 Costs

Monetary costs of implementation of a genetic diversity indicator are incurred during sampling, laboratory analysis, and data analysis. Costs of field sampling for DNA analyses are similar to costs to collect species assemblage and biomarker data, and will generally be shared with those indicators. McCormick and Peck (2000) estimate the cost to field a contracted crew of 4 people at $1200 per site visited. They estimate the cost of field equipment, including a backpack electrofishing unit, to be $3515 per crew, with a 15% annual maintenance and depreciation rate. Sampling for allozyme analyses requires the use of special storage containers and a regular supply of dry ice or liquid nitrogen. This may add an additional $50 per site in supply costs, plus approximately $400 in cold storage equipment. One possible consequence of the necessity for cold storage is that the crew may not be able to remain at remote sites

Figure 4-7. DNA quantitation is performed using a commercially available fluorescent nucleic acid stain that is detected with a fluorescent scanner.

Figure 4-8. Thermal cyclers are used for fragment amplification (RAPD, AFLP, microsatellites) and DNA sequencing assays.

Figure 4-9. A capillary-based, auto-loading genetic analyzer can perform both fragment analysis (AFLP, microsatellite) and DNA sequencing.

Table 4-2. List of standard and specialized equipment for different types of genetic marker studies. * optimal but not necessary.

All laboratories

1. Freezer- samples
2. Freezer- chemicals
3. Refrigerator
4. UV transilluminator
5. Ultrapure water source
6. Pipetters
7. Variable speed centrifuges
8. Microcomputer with statistical genetics analysis software

Standard Equipment - DNA laboratories

1. Separate sample preparation room/area (DNA extraction)
2. PCR room/area
3. Post-PCR room/area with photodocumentation and/or fragment analysis equipment
4. thermal cyclers
5. Agarose gel electrophoresis rigs
6. Microcentrifuges (10,000 RPM)

Specialized equipment- allozymes

1. -80 freezers
2. dry ice or liquid nitrogen and canisters
3. Starch gel electrophoresis rigs
4. Power supplies (to 350 V, 150mA)
5. Incubator oven.
6. Chiller (for cooling starch gels during run)*

Specialized Equipment - microsatellites

Microsatellite Development

1. Hybridization oven
2. Incubator oven
3. Automated DNA sequencer
4. Shaking incubator
5. Fluorescence detection system (see microsatellite screening equipment)

Microsatellite Screening

1. Fluorescence detection system, either
   (a) Acrylamide gel electrophoresis rigs, 1000 V power supply, fluorescence scanner, fragment analysis software, microcomputer
   (b) Automated DNA sequencer with fragment analysis software

Specialized Equipment - RAPD

1. Power supplies (to 350 V, 150 mA)
2. Specialized agarose gel electrophoresis rigs for recirculating buffer
3. Chiller unit (to 0º C) for cooling agarose gels during run*
4. Microcomputer with fragment analysis software

Specialized Equipment - AFLP

1. Fluorescence detection system, either
   (a) acrylamide gel electrophoresis rigs, 1000 V power supply, fluorescence scanner, or fragment analysis software, microcomputer
   (b) Automated DNA sequencer with fragment analysis software

long before samples must be shipped to the laboratory, which may increase sampling costs.

Laboratory costs are more variable, and depend greatly on both the type of molecular marker assessed and the technical skills of laboratory personnel. All estimates given here are provided with the caveat that they are likely to change rapidly. As with field sampling, labor is the greatest cost. Based on review of the EPA's pilot study that utilized the RAPD fingerprinting technique, it is estimated that a crew of four laboratory technicians can extract DNA from 95 samples, perform nine RAPD PCR assays per sample, electrophorese each PCR product individually, and size each of the fingerprint bands in a period of approximately 9 days. Assuming a typical sample size of 50-100 individuals per site, this indicates that a crew of four will require between one and two weeks to assay a single site. Supply costs, including enzymes, agarose, plastics and chemicals, are estimated at $9 per sample for nine RAPD assays. Equipment costs included two fluorimagers and associated computer hardware and software ($70,000 each) ten agarose gel units ($4000), chiller ($3000), and many smaller items (pipetters, stirrers, centrifuges, incubators, approximately $10,000). We assume an average depreciation of approximately 20%. The fluorimagers could reasonably be replaced with cheaper photodocumentation systems for less than $20,000.

Guidance on costs of other types of molecular marker studies can be obtained from estimates by commercial laboratories. It is assumed that this is a maximum cost estimate because a profit margin is built in, but depreciation costs are built into the estimates and these labs may experience higher efficiency than other labs. Allozyme electrophoresis typically costs between $10 and $40 per sample, with between 5 and 15 polymorphic loci (and often many more monomorphic loci) assayed. Costs to set up an allozyme laboratory fall in the range of $10,000 to $20,000. DNA sequencing typically costs $25-$40 per sample, for a sequence of up to 500 bp. It is often recommended that sequences be generated for both the upper and lower DNA strands, doubling the cost. This cost does not include the cost of DNA extraction and PCR amplification of the target locus, approximately $2 per sample. Automated DNA sequencers vary greatly in price and quality, but useful models can be obtained for between $70,000 and $130,000. Microsatellites and AFLP fingerprints that are assayed on automated DNA sequencer cost between $2 and $5 per sample run. For microsatellites, a single automated run may include between one and 8 distinct PCR reactions, increasing the efficiency of the assay. Costs of DNA extraction ($2) are not included.

Costs of marker development range from essentially zero for RAPD, AFLP, and allozymes to several thousand dollars for microsatellites. At least one commercial laboratory will guarantee production of ten usable microsatellite markers for any species for a cost of $20,000.

4.8 Summary Recommendations

Among the myriad different ecological indicators available for environmental assessments, the optimal niche of a genetic diversity indicator is to map patterns of population structure and to identify cumulative genetic changes in populations through spatial and/or temporal comparisons. Like other indicators of ecological condition, its value will be greatly enhanced if it is interpreted as part of a multi-indicator assessment. By its nature, genetic diversity is a generalized indicator of long-term changes in populations; it will be difficult to assign causation to any specific stressor. There are exceptions to this rule. For example, assays for specific allozyme genotypes may be developed as diagnostic indicators of particular classes of stressors (e.g., heavy metals) and, as more is learned about functional consequences of nucleotide variation at specific genes, DNA-level diagnostic indicators also may be developed. Presently, however, a genetic diversity indicator will be most useful as an integrative indicator of genetic effects imposed by multiple stressors. This suggests that the genetic diversity indicator will prove most

useful as one component of a multi-indicator regional or index-site assessment.

Although the point can be debated, of the currently available technology, microsatellite analysis, perhaps combined with mitochondrial DNA analysis, is likely to provide the most useful information per unit effort for both regional genetic diversity assessments and temporal genetic diversity monitoring. The US EPA's Molecular Ecology Research Branch employs this methodology for most current and planned assessments. Unfortunately, this strategy also requires relatively advanced instrumentation and technical expertise compared to other strategies. The EPA's ECBP pilot genetic diversity study (section 3.1) was conceived and implemented to use RAPD fingerprinting precisely because of concerns about the ability to transfer genetic diversity indicator technologies to end-users. Because RAPD fingerprinting is technically simple, it was assumed that it would have the greatest prospects for technology transfer. However, during the course of the pilot study it became clear that the trade-off that comes with ease of implementation is that of extreme sensitivity to minor variations in laboratory techniques. Similar concerns are echoed in the scientific literature (see Perez et al., 1997). Concerns about repeatability between different laboratories appear to be great enough to negate any perceived technology transfer advantages. Current work with AFLP fingerprints indicates much less concern with repeatability, but technology transfer is considerably more difficult than for RAPD analysis. Transfer of allozyme technology should not be difficult, but the needs for lethal sampling and methods to transport samples at ultra-cold temperatures limit its general application.

While complete protocols for development and analysis of microsatellite markers may be difficult to transfer to environmental labs, certain aspects of the analysis are relatively straightforward. As mentioned, methods for extracting DNA from animal tissues have been commercialized and are now sold by several vendors as kits. The quality and quantity of DNA extracted using commercial kits is typically very high. In addition, improvements in thermal cycler technology, together with packaging of PCR reagents as standard assay kits have greatly eased technology transfer of PCR. Several vendors now sell test kits for genotyping domestic animals and humans with fluorescent microsatellite DNA markers. This suggests that regional field labs could accomplish major parts of the genetic diversity analysis while one or two "core" molecular biology labs handle other aspects. A model for this approach is shown in Figure 4-10. The regional lab, perhaps with some advice from a genetic analysis lab, would design the assessment, which presumably will be performed in conjunction with other ecological indicators. A marker development laboratory can then be employed to develop a panel of markers that are appropriate to the assessment. The regional lab can then collect samples and prepare DNA for analysis. The actual PCR reactions can be performed by the regional lab or by the genetic analysis lab. The genetic analysis lab performs the genetic analysis and derives the genetic diversity interpretation. The regional and genetic analysis labs can then assess the ecological significance of the genetic diversity indicator data.

Under this scenario, the marker development laboratory could be a commercial laboratory or a laboratory internal to the EPA. The EPA currently has the required expertise for such a laboratory in the Molecular Ecology Research Branch of NERL. A laboratory internal to the EPA, possibly the same lab that is used for marker development, or a laboratory under contract to the EPA could perform the genetic analysis.

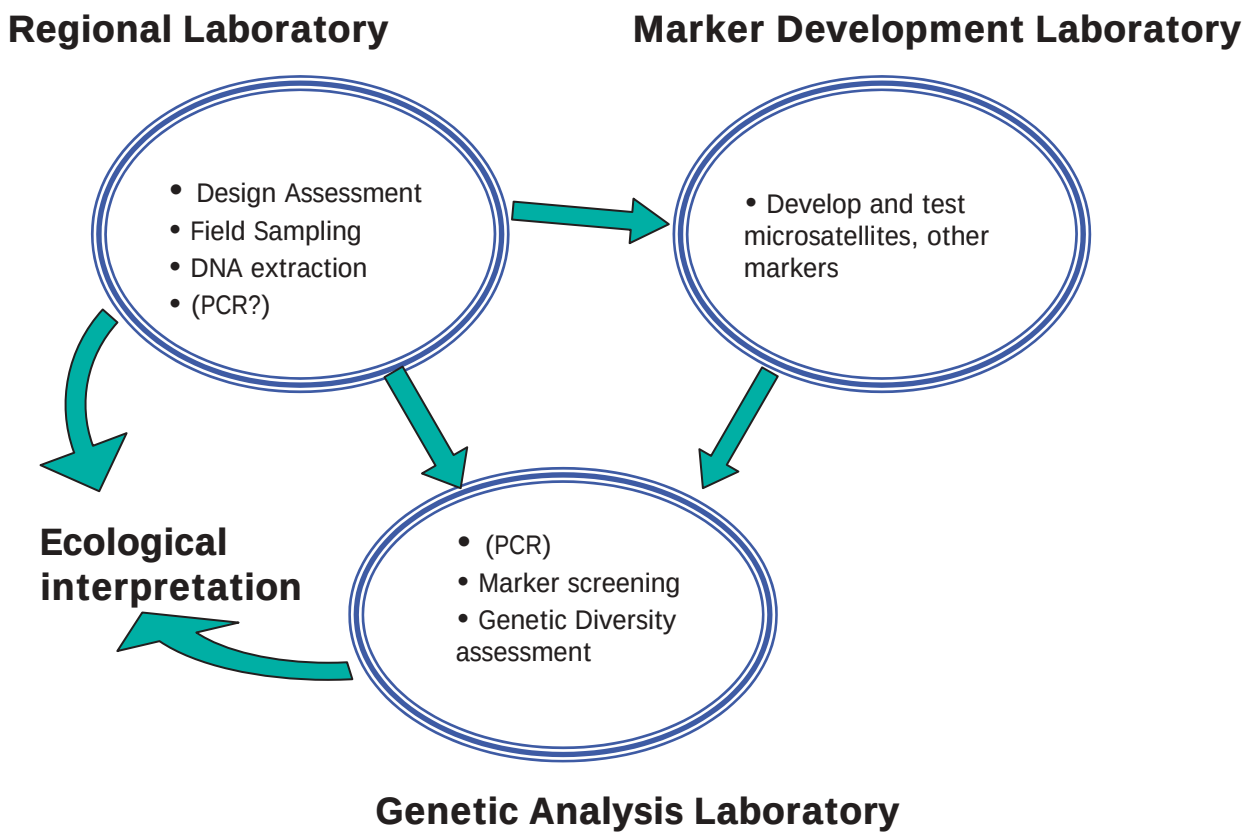

Figure 4-10. A model showing how three different labs, the regional field lab, a genetic analysis lab, and a marker development lab could interact to apply a genetic diversity indicator such as microsatellites or mitochondrial DNA sequences to an environmental assessment.

Glossary

Adaptation: (noun) A genetically determined trait that enhances the ability of an organism to survive and reproduce in its environment; (verb) the evolutionary process by which a population undergoes progressive genetic modification to increase its ability to survive and reproduce in a given environment.

Allele: one of several alternative forms of a gene that differs from other forms by a mutation in the DNA sequence.

Codominance: the condition where both alleles at a locus are expressed and influence the observed properties of the heterozygote.

Diploid: An organism that normally has two sets of chromosomes, and thus two copies of each gene.

Dominance: the condition in which one allele (the dominant allele) masks the expression of the other (the recessive allele), so that heterozygotes cannot be distinguished from individuals that are homozygous for the dominant allele.

Effective population size: The size of an "ideal population" (with a mathematically simplified breeding structure) that loses genetic diversity at a rate equivalent to the population under study. The effective population size is typically smaller than the census size because of differences in the breeding success of individuals, skewed sex ratios, variation in population size, and other factors. The effective population size provides a measure of how fast genetic diversity is being lost from the population under study.

Electrophoresis: The separation of DNA, RNA, or proteins by differential migration through a matrix in the presence of electric current. Electrophoretic mobility is determined by differences among molecules in size, charge, or shape.

Fitness: The genetic contribution by an individual's descendants to future generations of a population. Individuals with greater fitness have genotypes that are better matched to the environment than those with lower fitness.

Gene flow: The exchange of genes between generation via migration of individuals that will eventually breed.

Genetic diversity: Variation among individuals for some heritable trait. Intraspecific genetic diversity may be partitioned into at least two components: genetic diversity within populations and genetic diversity among populations.

Genetic drift: Stochastic change in allele frequency due to random variations in the contributions of breeding adults to the next generation. Since one consequence of genetic drift is that one or more alleles will not be passed to the next generation, genetic drift results in a decrease in genetic diversity.
Genetic marker: an easily discerned attribute or probe that indicates the genotype of an individual at a locus. Examples include molecular markers (usually DNA-based), cytological markers, and morphological markers

Genome: The complete set of genetic information contained within an individual

Genotype: The genetic make-up of an organism, typically with respect to one or a few genes of interest, as distinguished from its appearance or phenotype.

Haplotype: A mitochondrial DNA genotype. One of several forms of the circular mitochondrial DNA molecule that differs from other forms due to mutations in the DNA sequence.

Heterozygote: For diploid organisms, an individual whose cells contain two forms (alleles) of a gene, one derived from each parent.

Heterozygosity: The proportion of individuals in a population that are heterozygous (possess more than one allele) at a locus

Inbreeding: Mating among related individuals.

Inbreeding depression: The deterioration in a population's fitness and other traits due to a reduction in genetic diversity within populations.

Loci: Plural of locus.

Locus: A site on a chromosome where a gene or other measurable variation resides. The DNA at this chromosomal site may or may not have any functional significance to the organism.

Mitochondria: self-replicating organelles found in the cytoplasm of all eukaryotic cells that produce energy via oxidative phosphorylation.

Molecular marker: An easily discerned measure of the genotype of an individual at a locus based on molecular biological methods. Molecular markers are usually based on attributes of DNA, but may be based on RNA or protein.

Monomorphic: State in which virtually all individuals have the same genotype at a locus. It is a property of a locus in a population.

Neutral marker: A genetic marker derived from a locus that has a negligible effect on the ability of the organism to survive and reproduce.

Outbreeding depression: The deterioration in a population's fitness or other traits due to immigration of individuals from other populations that disrupts local adaptations.

Phenotype: The observable structural and functional characteristics of an organism that result from the interaction of the genotype with the environment; the outward appearance of the organism.

Philoptatry: The tendency of species or groups to remain in or habitually return to their native regions or territories. Organisms that are highly philopatric tend to stay in a defined geographic location.

Phylogeography: Reconstruction of biogeographic relationships, usually among populations within a species, by identifying the phylogenetic relationship between a genetic trait or set of traits in relation to geography.

Polymerase chain reaction (PCR): A process for the exponential amplification of a specific region of DNA using DNA primers that flank the region of interest and a DNA polymerase to catalyze the reaction.

Polymorphic: State in which individuals in a population have more than one genotype at a locus. It is a property of a locus in a population.

Polymorphism: One of several forms of a genetic characteristic at a locus in a population. Also, the presence in a population of two or more relatively common forms of a gene, chromosome, or genetically determined trait.

Population: A group of conspecific organisms that occupy a more or less well defined geographic region and exhibit reproductive continuity from generation to generation; it is generally presumed that ecological and reproductive interactions are more frequent among these individuals than between them and the members of other populations of the same species.

Recessive: An allele whose properties are not observed because they are masked by the expression of a dominant allele. Thus, it is only expressed in homozygotes.

Trait: Any aspect of the appearance, behavior, development, biochemistry, or other feature of an organism.

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Appendix 1: Laboratory and analytical procedures for RAPD analysis

DNA Extraction. For DNA purification, a portion of the frozen tail section (approximately 25 mg) was homogenized in a 1.5 ml microfuge tube containing 100µl PBSET (standard PBS, 100mM EDTA, 0.1% Triton X). 400µl of PBSET, 27µl Proteinase K (20mg ml^{-1}) and 30µl of 20% SDS was added to the homogenate. The tube was mixed by gentle inversion and incubated overnight at 65 °C. Following digestion, the preparation was centrifuged at 12,000 rpm for 10 minutes to remove particulate and undigested material. The supernatant was removed to a clean microfuge tube, incubated for 10 minutes at 91 °C to inactivate the Proteinase K, then allowed to cool to room temperature. 10 µl of RNAse(A) (20 mg ml^{-1}) was added to the supernatant and incubated for 30 minutes at 42 °C. 1 ml of ProCipitate was added to the tube, which was then mixed by gentle inversion on a rotator for 5 minutes and centrifuged at 14,000 rpm for 15 minutes at room temperature. To facilitate the separation of the DNA-containing supernatant and the semi-solid, protein-containing pellet, a dab of light PhaseLock gel (5'-3', Inc.) was added to the inside of the microfuge cap and the sample was centrifuged for an additional 15 minutes at 14,000 rpm at room temperature. The supernatant was carefully transferred to a 2.0 ml microfuge tube, after which an equal volume of chloroform-isoamyl alcohol (24:1) was addedto the supernatant and mixed by gentle inversion on a rotator for 15 minutes. The aqueous fraction was transferred to a new 1.5 ml microfuge tube and the DNA was precipitated by adding 1 µl of glycogen (20 mg ml^{-1}) and an equal volume of ice cold 100% isopropanol, mixing well by inverting, and incubating at -20 °C for 2 hours. The precipitated DNA was pelleted by centrifuging for 15 minutes at 15,000 rpm at 0 °C. The supernatant was removed and the DNA washed with ice cold 70% ethanol. The DNA was again pelleted by centrifuging for 15 minutes at 15,000 rpm at 0 °C. The supernatant was removed and the DNA washed again with ice cold 95% ethanol. The DNA was pelleted one last time by centrifuging for 15 minutes at 15,000 rpm at 0 °C. The supernatant was removed and the DNA pellet was allowed to air dry for 10 minutes at room temperature. After drying, the pellet was resuspended in 200 µl of 1X PCR buffer (10mM Tris-HCl, 50mM Kcl, pH 8.3), warmed for 15 minutes in a wet block at 65 °C, then incubated overnight at 37 °C in ensure complete resuspension. The DNA stock concentration was estimated by comparing the fluorescence of 1000-fold dilutions of the stock with known standards both prepared with PicoGreen fluorescent dye (Molecular Probes, Inc.) and scanned using a FluorImager 595 (Molecular Dynamics) with 488 nm excitation and 530df30 bandpass filter. Stock DNA was diluted to a working concentration of 1.25 ng μl^{-1} with 1X PCR buffer (10mM Tris-HCl, 50mM Kcl, pH 8.3).

PCR. RAPD analysis used was modified slightly from the original descriptions (Williams *et al.*, 1990; Welsh and McClelland, 1990). A total reaction volume of 25 µl was prepared by combining a 5 µl aliquot of the PCR master mix (2mM Tris- HCl (pH 8.5), 10 mM KCl, 3mM $MgCl_2$, 1 pmole RAPD primer, 2.0 units of Native *Taq* polymerase (Gibco BRL), 0.05% acetamide, 1mM dNTP mix) with a 20 µl aliquot of diluted DNA (1.25 ng μl^{-1}) in a 0.2 ml PCR tube (individual or strip) and mixing well. All PCR prep was done on ice. All media preparation and sample aliquoting was done using aerosol resistant pipet tips. Samples were amplified by 34 cycles of 45 seconds at 94 °C, 1 minute at 41 °C, 1 minute at 72 °C on a RoboCycler with Hot Top (Stratagene). Three decamer oligonucleotides (primers) were used to generate RAPD profiles; OPF-04 (5'-GGTGATCAGG-3'), OPL-02 (5'-TGGGCGTCAA-3'), OPL-05 (5'-ACGCAGGCAC-3'). Triplicate PCR reactions were performed, 95 samples plus 1 negative control per replicate.

Gel Electrophoresis. A 15% Ficoll 400/0.25% bromophenol blue solution was added to the completed RAPD products, along with 400 and 900 bp fragments and buffered salt solution; the additional DNA fragments provided in-lane standards to aid in sizing. Samples were arranged on a 20-lane agarose gel (1.65% $w\ v^{-1}$ containing 1X TBE [89mM Tris Base, 89mM Boric Acid, 10mM Na_2 EDTA-H_20]) such that each sample lane was placed adjacent to a molecular weight marker lane (500/100 bp ladder,

GenSura). Samples were electrophoresed at 7 °C, 6.8 V cm^{-1}, for 4.5 hours with continuous buffer (0.6X TBE) recirculation. The gels were subsequently stained in 1000ml of 1X TBE with 4 µg ml^{-1} of ethidium bromide for 30 minutes with constant agitation and then destained in water for 15 minutes. The gels were scanned using a FluorImager 595 (Molecular Dynamics) with 514 nm excitation and 590df30 bandpass filter, producing a visual image of the PCR products.

Image Analysis. Bands were declared using FragmeNT Analysis software (Molecular Dynamics) and then further edited visually to eliminate both false bands and those bands below a level of optical density that could be reliably scored. Sample band molecular weights were calculated by a point-to-point logarithmic interpolation method within FragmeNT using the standard lane nearest to the unknown.

Marker Identification. Despite the large number of molecular weight ladders, sizing errors inevitably occurred so fragment size alone is not a reliable indicator of band homology. Bands were classified into size groups (bins) using cluster analysis. These bins were further refined by discriminant analysis of band size and intensity characteristics. In excess of 200 such bins were identified; however, 53 bins that were most repeatably scored (based on comparison of the three replicates for each individual) were selected for analysis. The number of bins created ranged from 39 for primer F04 to 42 for primer L05. Bins in which the average band intensity was low (less than 0.5% of total lane intensity) were excluded from the analysis, as were bins in which the repeatability of scoring across replicates was poor. Once the bins were created, each bin was assessed for each individual to determine whether the bins were "filled" or empty. If a band was observed within the size bin for two of the three replicates scored then the bin was considered filled for that particular individual.

Similarity Analysis. RAPD fingerprints were compared between each pair of individuals using Lynch's (1990) similarity index (S), which is defined for two individuals, x and y, as $S_{xy}=2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of bins that are filled for both individuals, and N_x and N_y are the total number of bins filled for individuals x and y, respectively. The average similarity of individuals within a population ($\overline{S}_w$) provides an inverse measure of the genetic diversity within that population. Comparisons of average similarities among individuals in the same populations to average similarities among individuals in different populations ($\overline{S}_b$) provide a measure of population substructure. Hypothesis tests for significant differences in Sw's were constructed and tested according to the method of Leonard *et al*. (1999). Analogs of Nei's (1972) genetic distance estimate D' between each pair of populations were calculated following the method of Lynch (1991). Population substructure was estimated using the estimator of Wright's (1978) F_{ST} for DNA fingerprint data described by Lynch (1991). Estimates of the average number of migrants among populations were made using the approximate equilibrium relationship $F_{ST}= 1/(1+ 4N_em)$, where N_e is the effective size and m is the rate at which migrants are exchanged between populations per generation. N_{em}, therefore, represents the average (effective) number of migrants exchanged between populations each generation. Dendrograms relating genetic relationships among populations were assessed using unweighted pair group means analysis (UPGMA).

Quality Assurance. Several quality assurance measures were employed throughout this study. Incorporation of three replicates of each RAPD reaction into the design helped to ensure that RAPD fingerprints were highly repeatable. Each set of 95 fish to be processed was assigned a blind sample number based on the tube assignment of a 96-well PCR plate (8 rows [A-H] x 12 columns [1-12]). This sample number was carried through all steps of DNA extraction and quantitation, PCR, gel electrophoresis and scanning, FragmeNT analysis, and subsequent data handling. A SAS database was maintained to record the dates and any comments of all aforementioned steps. To avoid any PCR block position bias, the 95 samples and negative control within one PCR replicate were randomly assigned a position in the 96-well PCR block using a SAS-based random number generator. To avoid any gel lane position bias,

all samples and the negative controls for one PCR replicate of 3 primers (3 primers x 96 DNA samples/negative control = 288 PCR reactions per replicate) were randomly assigned a lane position on a gel (288 PCR reactions / 12 reactions per gel = 24 gels). A pre-printed form listing the load order of samples and specific gel numbers was used at the time of gel loading and any deviations in the assigned order were noted and changes were made to the database. Following electrophoresis and gel scanning, gel images were reviewed to verify that the samples were actually loaded in the lanes as assigned. The appearance of bands in a gel lane designated as a negative control was used to indicate contamination during the PCR preparation; any questionable replicate was discarded and repeated. Any PCR reactions that failed to amplify any bands were noted and an additional replicate PCR was performed. Any gels that produced a poor visual image due to problems associated with electrophoresis were run a second time with the balance of the PCR reaction. SAS-based programs were employed to compare the original gel load order assignments with those actually reported as an output from the FragmeNT Analysis; discrepancies were flagged and reconciled where possible.

Appendix 2: Laboratory and Analytical Procedures for AFLP Analysis

DNA Extraction. DNA was extracted from a section of tail fin (25-50mg) using a commercial kit (DNeasy, Qiagen) and quantitated using a fluorescently labeled nucleic acid stain (PicoGreen, Molecular Probes).

AFLP digestion/ligation/amplification. 200 ng of genomic DNA was restriction digested and ligated overnight at room temperature in a single 15 µl reaction as follows: 1X T4 ligase buffer (50mM Tris Hcl, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP), 50mM NaCl, 75 ng BSA, 5 units each Eco RI and Mse I, 100 Weiss units T4 DNA ligase, 30 pmol Eco RI adapter and 300 pmol Mse I adapter. The digestion-ligation products were diluted 10-fold with TLE [10mM Tris-HCL, pH 7.6, 0.1mM EDTA]. Preamplification reactions were performed using 5 µl of the diluted digestion-ligation product in a 20 µl reaction with standard EcoRI (Eco+G) and MseI (Mse+C) adapter primers (30ng each), 20mM Tris HCL, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM each dNTP, and 0.5 units DNA Taq polymerase. The following thermocycling profile was used for preamplification: 72°C for 2 min, 24 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, and ending with a final extension at 72°C for 30 min. The preamplification products were diluted 10-fold with TLE (10mM Tris-HCL, pH 7.6, 0.1mM EDTA). Selective amplification reactions were performed using a 5 µl of the diluted pre-amplification product in a 20 µl reaction with 20mM Tris HCL, 50mM KCl, 1.5mM MgCl2, 0.2mM each dNTP, 0.5 units DNA Taq polymerase, 5 ng MseC+1 primer, 15 ng EcoG+2 primer with a 5' fluorescein attachment. Ten EcoG+2/MseC+1 primer combinations were used for selective amplification PCR conditions for the selective amplification were as follows: 12 cycles of 94°C for 30 sec, 65°C for 30 sec (dropping 0.7°C per cycle), 72°C for 1 min, followed by 24 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min; a final extension at 72°C for 30 min completed the PCR.

Gel Electrophoresis. Selective amplification products were diluted 1:1 with sequencing load dye (deionized formamide, 0.5 mg/ml xylene cyanole); 28 samples and 5 lanes of a 10 bp size ladder were electrophoresed on 5% denaturing polyacrylamide gels in 0.5X TBE buffer at 25W for 1.3 hours. Gels were scanned using a Molecular Dynamics 595 FluorImager (488nm excitation, 530df30 filter) to visualize the fluorescein-tagged amplification products.

Fragment Analysis. Each lane was assigned a score based on visual quality (0 = no amplification, 1 = very few and/or light bands, 2 = only very intense bands are able to scored, 3 = dark bands are scorable, 4 = most bands are distinct over a range of intensity); lanes with a score <3 were not analyzed and those samples were subjected to repeat amplification and electrophoresis. Images were evaluated using Cross Checker (v 2.91) and a binary table indicating presence or absence for each sample/primer combo/marker was generated. A second Cross Checker evaluation was made by another analyst and the resulting binary scores were compared with the original interpretation. Replicates (DNA extraction through selective amplification) were done on 20% of the samples; binary scores from the replicate samples were used to determine reliability of a particular AFLP marker. Those markers whose binary scores were in disagreement, either between analysts or replicate samples were eliminated from the final analysis. Within and among population similarities were calculated; pairwise t-tests of the within population similarities were performed. A genetic distance matrix (Nei, 1987) based on the among- population similarities was constructed to test for evidence of population subdivision. A molecular analysis of variance (AMOVA, Excoffier *et al*., 1992) was performed to partition genetic diversity within and among populations.

Appendix 3: Laboratory and Analytical Procedures for DNA Sequence Analysis

Mitochondrial DNA amplification. Genomic DNA was amplifed with mitochondrial DNA primers for part of the cytochrome B region. Reactions were performed using 1.5 µl of diluted genomic DNA (25 ng/µl) in a 20 µl reaction with 50 µl each of cytochrome B primers GLU (5'-TGA CTT GAA GAA CCA CCG TT-3') and THR (5'ATC TTC GGA TTA CAA GAC CGA); 20mM Tris HCL, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM each dNTP, and 0.5 units DNA Taq polymerase (Gibco BRL). The following thermocycling profile was used for preamplification: 94°C for 5 min, 28 cycles of 94°C for 40 sec, 52°C for 30 sec, 72°C for 1 min, and ending with a final extension at 72°C for 5 min. The reaction products were diluted 1:1 with buffered load dye containing bromophenol blue and were electorphoresed in 1% agarose gels containing ethidium bromide in 0.6X TBE buffer at 250V for 2.5 hours. The band was visualized under UV light, excised from the gel, and purified using QIAquick kit (Qiagen). The resulting amplification product was quantitated using using a fluorescently labeled nucleic acid stain (PicoGreen, Molecular Probes) and diluted to a concentration of 2.5ng/ µl.

Mitochondrial DNA sequencing. Cycle sequencing reactions were performed in 20 µl using 1 µl of template (2.5 ng/ µl purified cytochrome B reaction), 2 µl ABI Ready Reaction mix (ABI BigDye Terminator [v.1] kit), 60mM Tris, 1.5mM $MgCl_2$, and 0.8 M GLU primer. Positive control reactions were similarly prepared using M13 primer and pGEM template. The following thermocycling profile was used for cycle sequencing: 25 cycles of 94°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. Excess terminator dyes were eliminated by isopropanol precipitation of the product. The sample was resuspended in deionized formamide and capillary electrophoresed (Applied Biosystems 3100 Genetic Analyzer; 50cm array, POP-6 polymer).

Sequence analysis. Sequences were reviewed in Sequence Analysis (v3.7, Applied Biosystems), edited and aligned by hand. Genetic structure was analyzed by AMOVA and minimum spanning networks using the software Arlequin (v2.0).

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