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Ecotoxicology and Population Genetics: The Emergence of "Phylogeographic and Evolutionary Ecotoxicology"

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Abstract. Genetics of ecotoxicology has recently emerged as a priority research field. The advent of polymerase chain reaction and molecular population genetics has made it possible to examine the genetics in even the smallest individuals. Although a potentially powerful technique, current approaches oversimplify the relationship of change in gene frequency to contaminant exposure. Many of these approaches cannot control for random correlation or accessory abiotic factors that impinge on the system tested. Indeed, the gestalt approaches of laboratory exposure or natural field experiments may ignore significant genome-level interactions that are important within a given system. At the very least, these approaches would benefit by a biogeographic survey of genetic variation to understand geographic microevolutionary patterns, or phylogeography, within a species to reduce spurious correlations and erroneous conclusions. Other single locus approaches can be chosen to enhance this approach if genetic/environmental interactions have been characterized for laboratory populations or for other model systems.

Keywords: phylogeography; ecotoxicology; population genetics

Introduction

The melding of traditional fields of toxicology and evolutionary genetics has received recent attention (Belfiore and Anderson, 1998; Bickham et al., 2000) with the realization that new molecular "tools" might prove useful in the assessment of contaminant impact on natural populations. Molecular approaches offer the potential to assess the genetic composition of populations and allow for correlative studies of population genetics and exposure to environmental contaminants. In some cases the molecular basis of, for example, pesticide resistance is known, and genetic surveys of a single locus can be used to estimate the magnitude of genetic change within populations attributable to specific chemicals (ffrench-Constant et al., 2000). Concomitant assessments of variation at other loci are useful indicators of how chemical stress affect loci not under direct selection. Unfortunately, many studies of "genetic ecotoxicology" (e.g., Lavie and Nevo, 1986; Murdoch and Hebert, 1994) do not

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focus on allelic variation at single well-characterized loci, but favor an alternative "gestalt" approach. Genetic diversity can be surveyed within populations by a variety of molecular means with the expectation that chemical contaminants depress the overall standing crop of genetic variability. The gestalt approach examines either: (1) multiple loci from individuals exposed to a contaminant and searches, *ad hoc*, for correlative changes at one or more of the loci, or (2) genetic markers from individuals at "clean" and "contaminated" sites and attributes genetic differences to contamination. To fully appreciate the ramifications of gestalt approaches, it is important to address population genetic processes and the underlying assumptions that are implicit in using such a paradigm.

Genetic variation in populations is molded principally by four factors: mutation, migration, genetic drift, and natural selection. Mutation is the ultimate source of new genetic variants, and the combined effects of the other three remaining factors interact to maintain or remove genetic variation from populations. The relative importance of the latter two forces is still quite controversial among population geneticists, but this dichotomy will not be addressed here. Mutation rates vary greatly among loci, but in general are small and not surprisingly have large errors associated with their estimation. A general assumption is that the normal mutation rate is low (approximately 10^{-6} substitutions per nucleotide site per generation). Statistical comparisons to test for increases in mutation rates associated with contaminant exposure are difficult to pursue since this approach requires untenable sample sizes for most species (unless the mutation rate is somehow altered, i.e., accelerated, by contaminant exposure itself). Migration between areas spread these mutations from the local to a more regional scale and increased migration leads to homogenization of gene frequencies among subpopulations.

A potentially potent mode of genetic divergence is a random loss of alleles in a process termed random genetic drift. Copies of alleles are limited to fewer individuals in smaller populations, so random individual events, e.g., premature death, unsuccessful mating, sterility, etc., have a greater proportional impact on the genetic constituency within a small, isolated population. Without migration (i.e., dispersal), subpopulations are genetically isolated from their nearest neighbor, and therefore are limited to the low mutation rate within the subpopulation for increases in genetic variability. Under such conditions, alleles are lost from the population at a rate that is related to the effective (= breeding) population size (N_e), and the loss of genetic variation is a natural (= random) outcome. Small populations (e.g., less than 100 individuals) are particularly susceptible to this type of genetic change.

In contrast, selection can be a potent force shaping the genetic composition of larger populations. The possession of one or more alleles by an individual within a population may confer an advantage or disadvantage to its survival and reproductive viability. In this case, alleles change in frequency because their presence in a genome is directly tied to the evolutionary success of an individual, a condition termed "fitness." Theoretically, a selection coefficient (s) larger than $1/2N_e$ is required for an allele's impact to be realized within a population of size N_e. Obviously in larger populations (like those of small estuarine invertebrates or some insects), small selection coefficients, e.g., $s \approx 0.001$, could have a large impact on the genetic constituency of the population.

The complex interaction of these forces in the context of a species' biogeographic distribution has become a field of study itself, called phylogeography (Avise, 1994). We now know that species ranges once considered homogeneous are often subdivided into distinct genetic subgroups through the interplay of historical factors and population genetic processes. Therefore, the gestalt approach of assaying levels of genetic variation within populations and unequivocally attributing these levels to a single source might be as untenable as estimating small changes in mutation rates. To understand effects of a stressor, e.g., pollution, on the genetics of a species, it is necessary to understand the interplay of mutation, migration, drift, and selection that creates the phylogeography, or genetic landscape, of a species' distribution.

Recent papers (Belfiore and Anderson, 1998; Bickham et al., 2000) have suggested new methods to examine the effect of environmental contaminants on this genetic landscape. Coincidentally, they suggest similar approaches to assess impact of contaminants on these natural populations. They argue that within a region, one needs to find contaminated and uncontaminated (= reference) sites and measure genetic variation within each subsystem. Impacts on the genetics of a population can be measured in terms of loss of genetic variability by direct measure (Bickham et al., 2000) or by elegant variance/covariance approaches (Belfiore and Anderson, 1998). Here, our oversimplification of their

strategies does not treat the fine points of their methods rather we detail some of the implicit assumptions they do not treat in order to refine a genetic/ecotoxicology research paradigm.

High mortality rates can have a distinct impact on genetic variation within a population. They impact to depress local gene frequencies via either selection or by random genetic drift. The case of selection is classic and straightforward: individuals with "sensitive" genotypes (comprised of susceptible alleles) are removed from a population by mortality or "effectively" by sterility. Selection of this type can be inferred through the examination of directly affected or linked loci. Of course, selection pressures might impinge on the genome as a whole, as in cases where diversity within and among loci appears beneficial (Hawkins, 1998); selection of this type is also inferable through examination of multiple loci, but its exact cause is not usually known (see below). Therefore, pressures of selection at the population level and individual level can be directly opposed, decreasing diversity at the population level while maximizing diversity at the individual level. Without an understanding of mechanism, it is impossible to predict changes in the population in response to stress, *a priori*.

The case of drift is more indirect. Populations lose genetic variation if the stress upon them and the resultant mortality is great. Severe reductions in population size leads to the loss of rare alleles via random genetic drift, but as opposed to selection, drift should affect all loci in the genome equally. The gestalt approach has application in this case, but showing a direct relationship between diversity and a particular "contamination" event is difficult, particularly given that the phylogeographic history of the species is usually unknown.

A model system for "phylogeographic and evolutionary ecotoxicology"

Research in freshwater and estuarine toxicology relies more and more upon invertebrate models of toxic response. In particular, there has been an expansion in recent years in the development of estuarine and marine meiofaunal-based models (Coull and Chandler, 1992 for review, Green and Chandler, 1996; Green et al., 1996; Chandler et al., 1997a,b; Kovatch et al., 1999). Yet for the majority of these and more standard EPA-recognized toxicological test organisms (e.g., *Mysidopsis bahia*, *Hyallela azteca*, *Palaemontes pugio*, *Ceriodaphnia dubia*), we know little regarding the phyleogeographic distribution of intraspecific genetic diversity.

The effects of toxicants on invertebrates present many unique challenges to the gestalt approach. The gestalt approach is implemented in one of two ways. First is the "genome correlation" approach. Test-animal A is exposed to a contaminant and the population is surveyed for multiple loci from within the controls and the exposed stocks. Any differences in genetic composition at any individual locus are due to selection on that locus. A recent example of this method is the exposure of scallops to copper where a positive correlation between multilocus heterozygosity and survival was found in young individuals (Troncoso et al., 2000) and the classic experiment of gastropods exposed to cadmium which showed heterozygotes for phosphoglucose isomerase were more susceptible to cadmium (Lavie and Nevo, 1986). In neither case is the mechanism of action known at the molecular level; it was assumed that heterozygosity at loci measured resulted in differential survival (albeit opposite ones in these cases) to metal exposure under laboratory conditions. Second is the "environmental correlation" approach. Individuals are sampled from "clean" and "contaminated" environments and genetic diversity is estimated. Differences in genetic diversity are compared in a multivariate approach to environmental parameters from the collection sites to explain reduction of genetic variation in terms of environmental variation, often comprising contaminant data (Murdoch and Hebert, 1994; Street and Montagna, 1996). Both approaches are flawed in that each has confounding factors and correlation of genetic change with contaminant load is usually equated with causation. The first case looks for a locus/susceptibility pattern among random loci when *a priori* knowledge of such a relationship for those loci is unknown. The second approach ignores the possible impact of differing phylogeographic histories between clean and contaminated sites or other non-contaminant related confounding factors.

A case study: *Microarthridion littorale* (Poppe 1881) in USEPA superfund sediments

The harpacticoid copepod *Microarthridion littorale* is a common inhabitant of muddy sediments in the

Southeastern US and has been considered to be an amphi-Atlantic species, with reported collections from the North Atlantic (Lang, 1948; Coull, 1977), southeastern US and Gulf of Mexico (Schizas et al., 1999), England, the Pacific northwest (McCall, 1992) and even Romania (Marcus, 1973). Given its broad distribution and availability, *M. littorale* has proved a useful organism for studies of sediment toxicity (Marshall and Coull, 1996; Chandler et al., 1997a) and basic meiofaunal ecology (Palmer, 1980; Morris and Coull, 1992 and references therein).

A broad geographic range such as that of *M. littorale* is usually indicative of high dispersal potential for a marine species (Scheltema, 1971, 1975). However, the estuarine dependence and lack of a planktonic dispersal stage for *M. littorale* would suggest low dispersal for the species. Certainly, other harpacticoid copepods (*Tigriopus*) in the rocky intertidal of the eastern Pacific have been shown to possess highly subdivided populations over short (10–100 m) spatial scales (Burton and Feldman, 1981; Burton and Lee, 1994). Given these conflicting factors, it was necessary to assess the population genetic structure of *M. littorale* to understand the evolutionary history of the animals in detail. As stated previously, it is crucial to know the phylogeographic history of a species before one can interpret the microevolutionary implications of their population genetics as they relate to pollutant-induced stress.

Relationships among *M. littorale* populations in the southeast Atlantic and northern Gulf coast were estimated (Schizas et al., 1999) from sequence data of two loci: mitochondrial cytochrome *b* apoenzyme (cyt *b*) and the first internal transcribed spacer of the nuclear ribosomal DNA (ITS-1). Phylogenies based on both genes were concordant. Three well-supported biogeographic groups were apparent for both genes analyzed: South Carolina, Florida, and Louisiana (Schizas et al., 1999). Even with no clear means of dispersal, populations are structured over hundreds of kilometers, rather than hundreds of meters for *Tigriopus californicus*, their California tidepool counterparts (Burton and Feldman, 1981).

Further examination of samples from the South Carolina genetic group indicated *M. littorale* consisted of distinct mtDNA lineages in cyt *b* (Fig. 1). Sequence divergence among 198 individuals was less than 4.3% but three divergent groups were discovered that differed by six to nine nucleotide changes without intermediate types present. An expanded survey on the frequency of these three lineages showed they co-occurred in seven

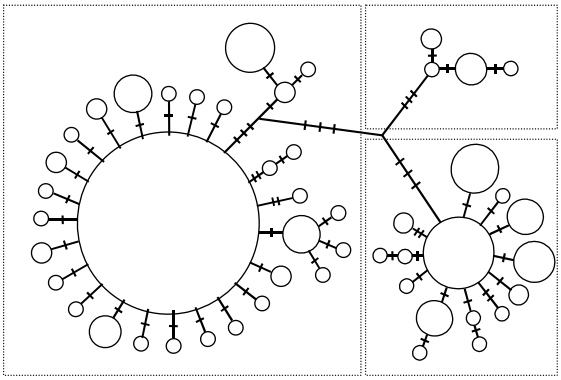

Figure 1. A minimum-spanning network of cytochrome b genotypes in *Microarthridion littorale* from North Carolina, South Carolina, and Georgia. Circle area is proportional to numbers of individuals with a given haplotype, ranging from 1 to 85 individuals. Hash marks across lines that join haplotypes indicate number of unique mutations separating haplotypes. The three boxes distinguish haplotype groups that can be discerned by restriction digest with *Dpn*II.

out of ten sampling sites. An analysis of molecular variance (AMOVA, Excoffier et al., 1992) indicated that a significant proportion of the total genetic variance could be partitioned among populations, although no significant correlation between geographical and genetic distance was uncovered. Genetic distance was, however, somewhat correlated ($p < 0.10$, Mantel test) with an estimate of total contamination from each sampling site (Schizas, 1999).

Although distributions of the three cyt *b* types can be partially explained by the presence of toxic contaminants in the sampled habitats, their apparent change in frequency cannot be causally linked to pollutants. In a laboratory study, representatives of the three lineages were collected from sediments of a very low contamination site (Buck Hall, South Carolina, USA, total sediment PAHs $< 40\,\mathrm{ng} \cdot \mathrm{g}^{-1}$) and were exposed to an aqueous pesticide mixture ($\sim LC_{90}$) containing additively equitoxic concentrations of chlorpyrifos and DDT (mixed isomers). Survival rates among the different haplotype groups were compared after a 24-h exposure in the laboratory. The most common haplotype group in South Carolina estuaries exhibited a significantly higher survival rate when exposed to these pesticides than the other two groups (Schizas et al., 2001). The two less frequently occurring groups, taken together, had significantly reduced survival rates in the presence of pesticides $P < 0.05$, Mann–Whitney U-test). These laboratory results mirror the frequency variation in mtDNA types in field-collected individuals

from clean and contaminated tidal creeks on the South Carolina coast.

In short, there is significant genetic variation in *Microarthridion littorale*, even though it was previously considered a widely distributed species (i.e., more likely to be homogeneous across its range). To blindly attempt to correlate environmental toxicants with genetic patterns without first understanding the geographic variation inherent in this species could have produced disastrous and confounding conclusions. Even within a single collection site, the disparate haplotype lineages could have been yet another possible source of confusion if the baseline of molecular variation among haplotypes had not been established, *a priori*. We, also, were surprised by the field correlation of haplotype distribution in response to pollution. Since, mtDNA is within an organelle and much of its variation is at the third position of the codon, it is usually considered neutral with respect to selection. Also compared with published data for other species, the *M. littorale* network of haplotypes (Fig. 1) is unusual. In our case, there is no common ancestral haplotype to the three groups (Fig. 1). Such a divergent pattern in haplotype diversity within a population is difficult to explain, genetically, for a single species.

A third approach—functional comparative genomics

Evolution of resistance to pesticides and other contaminants are known to occur in many arthropod species. In the case of pesticides, several species have been noted to gain population resistance by the allelic replacement of the common type with a resistant type differing in a single amino acid (ffrench-Constant, 1993). Such a mechanism provides a powerful model for analyzing similar loci in other arthropod systems for their response to pesticides. In such a case, the actual effect of an environmental stressor on the locus is known and a causal link to selection by that stressor can be readily made. Since small marine invertebrates can have very large census sizes (e.g., 10^5–10^6 individuals $\cdot$ m^{-2}) and presumably large effective (= breeding) population sizes, drift is effectively eliminated and changes in gene frequency result primarily from selection (i.e., selection coefficients > $1/2N_e$). Another aspect of large effective population size is that invertebrates are capable of maintaining greater genetic diversity at the resistance loci, so their populations have the potential to respond genetically to stresses. Therefore, for sediment systems, meiofaunal invertebrates seem a logical source in which to test the evolutionary impacts of environmental contaminants.

To test this model of resistance, we have begun to isolate GABA receptor loci from meiofaunal species. Thus far, we have portions of a β-like subunit $GABA_A$ receptor from the copepod *Microarthridion littorale* and the estuarine nematode *Cylindrotheristus miamiensis*. The β-subunit is the class that contains homologues to the *rdl* locus in dipterans, which confers resistance to cyclodiene pesticides such as dieldrin and endosulfan. We are beginning to develop primers to amplify this gene from single individuals via PCR to make population screening possible. In this case, we can test for direct selection of allelic variants in response to cyclodiene pesticide exposure. Given the confounding effects of drift and selection on alleles in general, emphasis on a single locus approach where there is strong evidence of gene/contaminant interaction provides the strongest model system currently possible for genetic assessment of environmental impacts on natural populations.

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