

FINAL REPORT

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Title: A Model of Insect Genetic Diversity in the Mobile/Tensaw River Delta

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Objectives of the research project:

Biodiversity of the Delta : The Mobile/Tensaw Delta, the nation's second largest river delta, harbors a wealth of biological diversity. Much of this 250,000 acre area of cypress-gum swamps and bottomland hardwood forests is relatively undisturbed. In addition, a staggering 15% of the nation's water (excluding Alaska) runs through the Delta. While we know the Delta is one of the country's largest depositories of biodiversity, little of the biota has been documented. Because our knowledge of this biota is, at best, poor, critical management decisions become little more than educated guesses. Also of even greater concern is how our ignorance of the Delta's biota undermines our estimates of its health. Current assessment of the estuary's health has been based largely on physical and chemical parameters, however, assessment without reference to the existing biota will inevitably produce erroneous conclusions (Paulsen et al., 1998). For example, based on chemical and physical parameters, the Delaware Department of Natural Resources concluded that 87% of its streams fully supported aquatic life (DNREC, 1994). However, based on a survey of benthic macroinvertebrates it became clear that only 13% of these streams actually were able to support the aquatic biota (Maxted, 1997). Although dramatic, such errors are common when habitat assessment is based solely on physical and chemical criteria (Paulsen et al., 1998).

Clearly, a well planned and detailed inventory of the Delta's biota is justified and currently ACES is funding some of these preliminary surveys. A common measure of biodiversity for such surveys is species richness. Unfortunately, reliance solely on species level taxonomic precision can lead to a simplistic overview of the available biodiversity. Hughes et al. (1997) estimated that there are 220 genetically distinct populations per species. A long-standing challenge for ecologists has been to link local ecological and evolutionary processes with higher order ecogeographic features (e.g., Thompson, 1999). The study of metapopulations shows that outcomes of a particular species interaction or the trajectory of a particular population is not homogenous over a large spatial scale; rather, outcomes vary among local populations (e.g., Thompson, 1999; Hughes et al., 1997). Thus, ignoring genetic data can lead to a significant underestimation of biodiversity, species movements, deme limits, and available 'seeds' for restoration or recolonization of impaired communities.

Genetic diversity and gene flow: It is generally accepted that ecosystems with high biodiversity are healthier than similar ones with low biodiversity (e.g., Baskin, 1994). Indeed, biodiversity studies are now conducted to assess ecosystem health. Extending the same logic, it would seem obvious that intraspecific diversity (i.e., genetic diversity)

and gene flow are equally important measures of ecosystem health (e.g., Beaty et al., 1998).

Intraspecific genetic variation can be used as a sensitive tool for the observation of habitat disturbance, either as a predictive tool based on declining genetic diversity, or as a post-hoc measure of otherwise unobserved events. For example, Golini and Rothfels (1984) identified genetic changes in a population of the black fly *Eusimulium euryadminiculum* (Diptera: Simuliidae) in the Chalk River, (Ontario) near a nuclear research facility, in comparison with sample studied twenty years earlier. Extensive DDT usage in the intervening years apparently extirpated this non-target population, which was later replaced with conspecifics.

High levels of gene-flow maintain genetic diversity within a species by effectively increasing population sizes, and hence decreasing the opportunity for “island effects” (e.g., Begon et al., 1996) which might result in local extinction. Conversely, high levels of homozygosity (i.e., an excess of individuals carrying two copies of the same allele, rather than two different alleles) in an otherwise polymorphic species indicates localized inbreeding, which can reflect habitat disruption resulting in genetic islands. Such small, isolated populations, are more susceptible to stochastic events resulting in extirpation.

Gene-flow and heterozygosity studies involve the genetic comparison of conspecific samples, the calculation of average heterozygosity, and the use of F statistics (Nei, 1977, 1978) to infer the relative amount of genetic contact among the populations represented by the samples. Such studies are extremely important for gauging the amount of movement that has historically occurred with a species, and the current status of populations with respect to their relative isolation from conspecific populations.

Detection of gene-flow barriers and localized inbreeding depends upon the existence of sufficient genetic diversity within a species so that heterozygotes are likely to be detected in reasonable sample sizes. Useful levels of genetic diversity within a species cannot be assumed. For example, many black fly populations with huge population sizes exhibit levels of allozyme diversity lower than inbred laboratory rat populations (Feraday and Leonhardt, 1989). For that reason, this project focuses on performing a genetic survey of several species of aquatic insect in order to identify species with sufficient levels of genetic polymorphism to be useful as models of genetic diversity in the Delta. This study will assess the levels of polymorphism in nuclear genetic loci. While the maternally inherited mitochondria DNA has proven very useful in studies of well-defined genetic systems, only examination of nuclear DNA can reveal heterozygotes, which must be observed to calculate average heterozygosities and F statistics. Furthermore, “useful” species must be relatively abundant, widespread throughout the Delta area, easily identifiable, and highly vagile to avoid confusion between “natural” inbreeding because of limited population contact/movement and island effects imposed by habitat degradation.

Significance and utility of gene-flow studies: The goal of this project is to identify model organisms, with appropriate DNA markers, for use in subsequent gene-flow studies in the Delta. Gene-flow studies are of special importance in determining the limits to populations and meta-populations because they measure the genetic relatedness

of samples. Direct observation of the movements of individuals (e.g., in mark-release experiments) cannot demonstrate genetic continuity among sampling sites. This problem is especially acute in the study of insect populations, since the routine observation of mating of the marked individuals is virtually impossible.

A thorough understanding of the population structures present in a region of special biological interest is critical to the design of rationale conservation strategies. For example, if populations of a vagile species in a given region show a high degree of gene-flow among them, then a plan of scattered conservation sites may prove highly effective in preserving genetic diversity. If, on the contrary, little gene flow is shown among samples then large, area-wide reserves would prove a more effective conservation strategy.

In summary, this project will allow the development of genetic tools to assess the population structure in aquatic insects of the Delta region, and provide preliminary data on gene-flow. A sound understanding of population structure is an absolute requirement for the design of robust conservation measures and so the results of this study and subsequent work, will directly aid in the conservation of biodiversity in the Delta.

Specific goals:

- 1) The identification of candidate species based on abundance, range with the Delta, ease of identification to species level, and high levels of vagility.
- 2) The development of DNA extraction techniques for these species, which will yield usable amounts and quality of DNA for subsequent genotyping experiments.
- 3) Assessment of nuclear loci for polymorphisms.

Summary of findings

Candidate species:

Candidate species were selected based on their abundance, range and ease of identification to the species level. Three highly vagile species were chosen as potential models for gene flow studies:

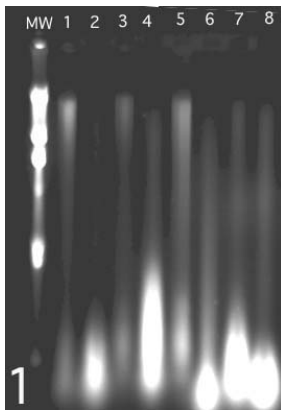
- 1) *Rantra nigra* (water scorpion; Hemiptera: Nepidae)
- 2) *Pleocoris femoralis* (Hemiptera: Necoridae)
- 3) *Eurythermus simplicolis*. (Dragon fly ; Odonata: Libellulidae)

DNA extractions:

Four basic DNA extraction protocols, plus variants on each, were performed with each of the candidate species. The four basic methods tried were:

- 1) Standard SDS extraction method (*cf.* Sambrook et al., 1989) (also supplementing the detergent with non-ionic detergents)
- 2) The black fly DNA extraction method of Brockhouse et al., (1993).
- 3) The Quiagen DNeasy Tissue kit (two alternate protocols: “insect” and “mouse tail”)
- 4) Rapid DNA extraction of Robertson (1993) (plus supplementing with non-ionic detergents)

Two methods for fixing specimens were also assessed. DNA was extracted from all three species from both ethanol fixed specimens (fixation protocol following Brockhouse et al., 1993) and from freshly frozen individuals. *Ranatra* yielded more DNA from ethanol fixed specimens, *Pleocoris* yielded more DNA from fresh specimens, but all the DNA was unusable in PCR assays (see below) and ethanol fixed *Eurythermus* yielded no DNA at all. We further investigated the role of fixation in DNA preservation for *Eurythermus* (Bennet et al., in preparation). Intriguingly, *Eurythermus* survives submersion in 95% ethanol for extended periods of time (> 1 hour). However, significant DNA degradation occurs within 15 minutes. By the time the specimen has been killed by the ethanol there is essentially no intact DNA left in its cells. All DNA extractions from specimens of dragonfly and damselfly housed in the University of South Alabama Insect collection showed that no DNA was present in the tissues of the fixed specimens (see Figure in Appendix A).



Preliminary DNA extraction experiments were conducted with 5 individuals per species. Success was judged on the production of DNA >10kb long as judged by agarose gel electrophoresis that was PCR-amplifiable. The successful protocols are shown in Table 1. A “+” indicates that DNA meeting the selection criteria was isolated, a “-“ indicates that it was not. Figure 1 shows example DNA extracted from *Ranatra nigra* using the method of Brockhouse et al., (1993). Seven of the eight individuals shown on the gel yielded DNA worth further assessment in PCR assays (Saiki et al., 1988). Once DNA extraction conditions were established, all available specimens of each species were processed according

to the most successful protocol (see Table 1).

Table 1			
Species	Brockhouse et al	Method	
		SDS or CTAB lysis followed by GeneClean kit (Bio 101)	Qiagen DNeasy DNA Purification kit
<i>Ranatra nigra</i>	+	-	-
<i>Pleocoris femoralis</i>	-	-	-insect protocol +Mouse tail protocol (DNA unstable)
<i>Eurythermus simplicolis</i>	-	-	-insect protocol +Mouse tail protocol

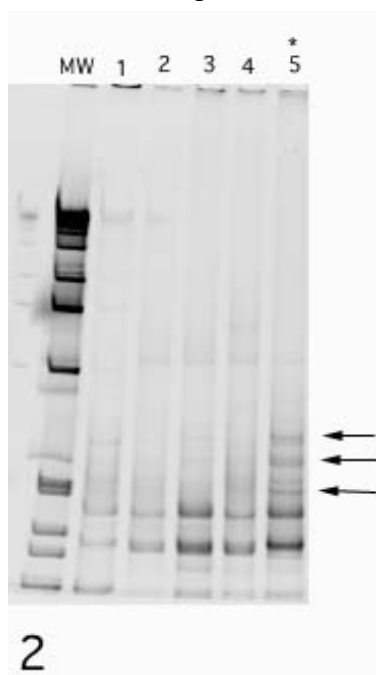
PCR:

Our anticipated strategy was to design PCR primers for each candidate species, based on DNA sequences in public data banks. However, none of the candidate species have sequences represented in the data banks, nor do they have close relatives (same family) included in the data banks.

To overcome this problem, we used primers from a collection of general (predominantly insect-based) nuclear DNA loci, compiled by Bernie Crespi (Simon Fraser University), and available from the University of British Columbia Nucleic Acid-Protein Service Unit (hobbs@ubc.ca). We concentrated on the use of EPIC primers (intron spanning primers; Palumbi, 1996) Primer pairs were tested for the ability to produce PCR products with 0.1 ug of template DNA. The loci that were produced were then assessed for polymorphisms (see below). In general terms, both *Ranatra* and *Eurythermus* yielded DNA that could be amplified by the PCR process, but few *Pleocolis* individuals yielded amplifiable DNA. We were able to amplify some individuals through the addition of a large amount of bovine serum albumin to the PCR reactions, suggesting the presence of a potent PCR inhibitor in the tissues of this species.

Polymorphism scan:

The PCR products obtained from the candidate species were assessed for sequence polymorphisms by a modification of the heteroduplex method (Cotton, 1997). Briefly, PCR products from multiple individuals were mixed together, then heated and cooled to create heteroduplexes. This mix was then analyzed by polyacrylamide gel



electrophoresis. The presence of a polymorphism within the sample will result in the presence of multiple bands. PCR products of single individuals were also then analyzed by polyacrylamide gel electrophoresis; heterozygotes will show multiple bands because a heteroduplex between the two alleles forms as a result of the PCR process. Figure 2 shows a sample gel of *Eurythermus simplicolis*. One individual (#5, marked by an asterisk) is a heterozygote. The arrows show the location of the extra bands produced by the heteroduplexes. Thus, this locus (coding sequence and intron of Elongation Factor 1-alpha;) shows significant levels of polymorphism even within a small sample. Table 2 gives a summary of the number of loci assessed per species.

Table 2

Species	Number of loci assessed	Number of polymorphic loci
<i>Ranatra nigra</i>	10	3 ; In progress
<i>Pleocoris femoralis</i>	10	-; In progress
<i>Eurythermus simplicolis</i>	10	5

Students trained:

Three undergraduate students and one graduate student were given practical laboratory training in DNA extraction methods, DNA cloning, agarose and polyacrylamide gel electrophoresis, image analysis and genotyping. This was done in the context of directed studies projects as a core component of this project.

Directed studies students:

Theresa Saultes (summer 2002; B.S. directed studies): *Ranatra nigra*

Samar Nasar (fall 2002; B.S. directed studies) *Ranatra nigra*

Chante Laster (fall 2003; B.S. directed studies) *Pleocoris femoralis*

Elizabeth Bennett (summer and fall 2003; M.S. directed studies) *Eurythermus simplicolis*

Conclusions

The most promising species for insect genetic diversity in the Mobile/Tensaw River Delta are *Ranatra nigra* and *Eurythermus simplicolis*. A significant, but addressable problem is that *E. simplicolis* must be purpose-collected as specimens fixed in ethanol contain no DNA due to rapid DNA degradation. Specimens frozen in the field on dry-ice, bisected and immediately fixed in cold (-20°C) 95% ethanol yield DNA that is usable for PCR and subsequent experiments..

Future Studies

There is an intriguing lack of insect diversity within the Delta region (McCreadie, unpublished data). Far more species of a given group can be found in a small region outside the Delta than within it. For example, more species of dragonfly have been found on the main campus of the University of South Alabama than in the Delta. There are at least two possible causes for this: environmental degradation in the Delta depleting species diversity, or unusual environmental conditions to which few species are adapted. In the former case, genetic diversity of surviving species would show a significant reduction, while in the latter case genetic diversity should match that found in conspecific populations in “non-impacted” locales. Comparative measures of genetic diversity in either *Ranatra* or *Eurythermus* would illuminate this important issue.

The rapid degradation of the DNA in specimens housed in the University of South Alabama Arthropod Depository highlights the need for a DNA bank, in which purified DNA is archived for future genetic work. Such work might include genetic diversity studies to monitor habitat degradation or the emergence of new species of pathogens. This project has provided the preliminary data for a grant proposal to establish a DNA bank.

References

- Baskin, Y. 1994. Ecosystem function of biodiversity. *BioScience* 44: 657-660.
- Beatty, B.J., Black W.C IV., Carlson, J.O., Clements, W.H., DuTeau, N., Harrahy, E., Nuckols, J., Kenneth, E., Olsen, K.E., and Rayms-Keller, A. 1998. *Environ Health Perspect* 106: 1395-1407.
- Begon, M, Harper, J.L ., and Townsend, C.R. 1996. *Ecology: individuals, populations and communities*. 3rd ed. Blackwell Science, Cambridge, Massachusetts.
- Black, W.C IV., Baer, CF., Antolin, MF., and DuTeau, NM. 2001. Population genomics: genome-wide sampling of insect populations. *Annu. Rev. Entomol.* 46: 441-469.
- Brockhouse, C.L., Vajime, C.G., Marin, R., and Tanguay, R.M. 1993. Molecular identification of onchocerciasis vector sibling species in black flies (Diptera: Simuliidae). *Biochem. Biophys. Res. Commun.* 194: 628-634.
- Cotton, R.G.H. 1997. *Mutation detection*. Oxford University Press. Oxford.
- DNREC. 1994. 1994 Delaware watershed assessment report. Volume 1. Executive summary. Dept. Nat. Resources Environ. Contr. Dover, Delaware.
- Feraday, R.M., and Leonhardt., K.G. 1989. Absence of population structure in black flies as revealed by enzyme electrophoresis. *Genome* 32: 531-537.
- Golini, V.I. and Rothfels, K. 1984. The polytene chromosomes of the North American blackflies in the *Eusiumulium caninicolium* group (Diptera: Simuliidae). *Can. J. Zool.* 62: 2097- 2109.
- Hughes, J.B., Daily, G.C., and Ehrlich, P.R. 1997. Population diversity; its extent and extinction. *Science* 278: 689-692.
- Maxted, J.R. 1997. Biology, probability and the obvious. *Human Ecol. Risk Assess.* 3: 995-965.
- Nei, M. 1977. F-statistics and analysis of gene diversity in subdivided populations. *Ann Hum. Genet.* 41: 225-233.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distances from a small number of individuals. *Genetics* 89: 583-590.
- Palumbi, S. 1996. The polymerase chain reaction. **In:** *Molecular Systematics*. 2nd edition. Sinauer, Sunderland.
- Robertson, H. 1993 The mariner transposable element is widespread in insects. *Nature* 362: 241-245.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich., H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Thompson, J.N. 1999. Specific hypothesis on the geographic mosaic of co-evolution. *Am. Nat. Supl.* 153: S1

Publications /presentations

Brockhouse, C.L. 2003. Rapid methods of detecting DNA polymorphisms. 1st Annual meeting of the North American Black Fly Association. Archbold, Florida. (symposium presentation)

Bennet, E., Brockhouse, A., McCreadie, J., and Brockhouse, C.L. 200-. Rapid DNA degradation limits recovery of DNA from archived insect specimens. *Insect Molecular Biology*: in preparation. Anticipated date of submission April 2004(see Appendix A).

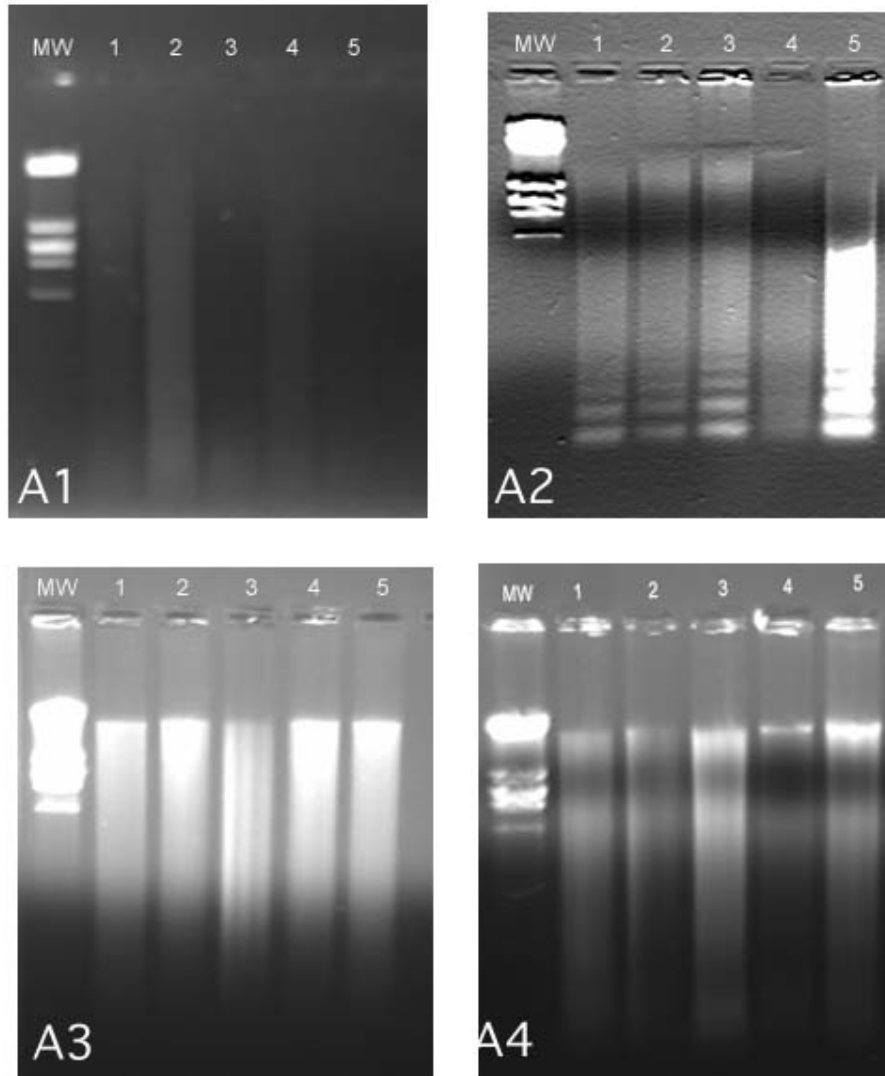
Richard, G., Brockhouse, A., McCreadie, J, and Brockhouse, C. 200-. A scanning method for genetic polymorphisms in natural populations. *Genetics*. in preparation. Anticipated date of submission: July 2004.

Supplemental Keywords

Aquatic, survey, genetic polymorphism, population structure, genetics, insects

Appendix A:

Figure from draft of Bennet, E., Brockhouse, A., McCreadie, J., and Brockhouse, C.L. 200-. Rapid DNA degradation limits recovery of DNA from archived insect specimens.



DNA extracted from *Eurythermus* fixed by different protocols. A1 shows DNA from specimens housed in the insect collection. A2 shows extremely rapid DNA degradation after only 15 minutes in ethanol. A3 and A4 show DNA extracted under different fixation protocols.