ABSTRACT

Title of Document: GENETIC DIVERSITY AND PERSISTENCE OF MAYFLY POPULATIONS IN DISTURBED HEADWATER STREAMS

Laurie Constance Alexander, Ph.D., 2007

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Movements of individuals shape the spatial structure of populations and play an important role in their persistence. For aquatic insects with winged adult stages, properties of the terrestrial landscape influence in-stream habitat quality and, in naturally patchy habitats such as dendritic stream networks, connectivity among habitat patches. Connectivity here refers to the population dynamics dependent on migration and gene flow among insect populations in semi-isolated stream segments. When populations are spatially connected, effects of local disturbance (e.g., habitat loss or degradation) can have a ripple effect, ultimately altering regional processes that reflect back to the local patch. But since regional and local population dynamics occur at different rates, detrimental effects of local disturbance are often not detected by biomonitoring efforts at the patch level until they have rippled through regional processes, by which time large-scale population extinction risk may have become unacceptably high.

My dissertation examines the effect of local and regional disturbance on the population density, genetic structure, genetic diversity, and persistence of mayfly populations living in forested and deforested headwater streams in the Central Piedmont region of Maryland and Virginia. I sampled populations of the mayfly *Ephemerella invaria* (Walker) in 24 first-order streams across 9 headwater stream networks. The sampling period (2001-2004) spanned a regional drought during which some of the streams went dry. Thus I was able to look at the interaction of local deforestation and stochastic regional disturbance in my study system.

In summary, my results indicate that in these mayfly populations:

1. Historically, long-range dispersal of *Ephemerella* occurred at levels sufficient to maintain gene flow across major watersheds, indicating excellent passive or active dispersal capability in these insects.
2. Deforestation of small watersheds decreases the rate of stream re-colonization and the recovery of prior population densities following a major disturbance.
3. Deforestation is correlated with loss of population genetic diversity.
4. Highly differentiated migrants represent a disproportionate share of the diversity in some mayfly populations.
5. Stochastic regional disturbance (e.g., drought) interacting with local disturbance (e.g., small scale watershed deforestation) can increase population extinction risk.

GENETIC DIVERSITY AND PERSISTENCE OF MAYFLY POPULATIONS IN DISTURBED HEADWATER STREAMS

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
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Preface

This dissertation contains three research chapters presented in manuscript form with abstract, introduction, methods, results, and discussion, followed by tables and figures. A single reference section occurs at the end for literature cited throughout the dissertation.

Dedication

For my parents, John Edward and Constance MacMillan Alexander; and my husband, Mark I. Mendelsohn.

Acknowledgements

I thank my dissertation advisors, Bill Lamp and Dave Hawthorne, for introducing me to aquatic entomology and inspiring me to work with mayflies in small streams (Bill), teaching me the methods of population genetics to answer questions about mayfly ecology (Dave), and making the work so much fun.

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Lastly I thank my brothers and sisters, John (Sandy), Sally, Peter, Susan, and Robert; their spouses and partners Bonnie, John, Elizabeth, Steve, and Jeanne; their children Tony, Alexis, Cameron, Nicol, Ben, Tilly, Flo, Adrienne, Kaylie, and Eric; my Airedales Chort, Sparky, Harry, and Wendy; and of course, the mayflies, for bringing so much joy into my life.

Most of all, to my wonderful husband Mark Mendelsohn: thanks, Hon.

Table of Contents

List of Tables

List of Figures

CHAPTER I: A Molecular Phylogeny of Closely-Related Species in the Genus *Ephemerella* (Ephemeroptera: Ephemerellidae)

Abstract

A molecular analysis of genetic lineages in the mayfly genus *Ephemerella* (Ephemeroptera: Ephemerellidae) was conducted using mitochondrial DNA (mtDNA) markers in comparison to species taxa delineated by morphologic characters. In a recent systematic revision of the genus, eight species including *E. inconstans*, *E. rotunda*, and *E. floripara* were synonymized with the widely distributed *E. invaria* based on morphology. Maximum likelihood and maximum parsimony analyses of mtDNA sequences placed one synonym, *E. inconstans,* with *E. invaria* in a well-supported clade (92%, 1000 bootstrap replicates). However, *E. invaria* samples were grouped in a nested clade (84% bootstrap support) and average Kimura 2-parameter (K2P) genetic distance between lineages (5.2%) was high relative to K2P distance within lineages (1.3%). The phylogenetic relationships of synonyms *E. rotunda* and *E. floripara* are not well resolved by this analysis but estimates of mean genetic distance from the *E. invaria* clade were high for both (8.7% and 11.2% K2P respectively). Cryptic diversity was revealed in species other than *E. invaria*. Samples identified as the widespread species *E. dorothea* were placed in two clades (90% and 70% bootstrap support respectively) with overlapping geographic ranges. Mean K2P genetic distance between the clades is 12.9%. An even larger genetic distance (18.7% mean K2P) was discovered between the eastern and western populations of *E. excrucians*; and western samples of one outgroup, *E. aurivillii,* were so genetically distant from all other species (mean 31.4% K2P) that doubt about its congeneric status is raised. While these results reveal high genetic diversity in and among morphologically

similar taxa, they do not support use of a "DNA barcoding" approach for identifying species in this genus, as evidence of incomplete mtDNA lineage sorting and retention of ancestral polymorphism also was found.

Introduction

Morphological species taxa often mask biological diversity of genetic lineages and infraspecific taxa that are genetically or ecologically, but not anatomically, distinct (e.g., Williams *et al.* 2006, Monaghan *et al.* 2005, Paterson 1991). Integrated use of molecular, morphologic, and biogeographic data in empirical systematics has expanded our knowledge of hidden diversity in many groups (Bickford *et al.* 2006) and is changing traditional approaches to confronting species uncertainty (Hey *et al.* 2003). Here I present results of a molecular analysis of genetic lineages in the mayfly genus *Ephemerella* (Ephemeroptera: Ephemerellidae) using mitochondrial DNA (mtDNA) markers.

Morphological similarity of mayflies in the genus *Ephemerella* has made identification of species problematic even for taxonomic experts, who disagree about the identification of specimens and the validity of current species taxa. In 2001 I started a population-level study to look at effects of land use on genetic diversity of a geographically limited mayfly species, *Ephemerella inconstans* (Traver), selected for its life cycle and preference for colonizing headwater streams in the piedmont zones of Maryland and Virginia. Two years into the study, however, Jacobus & McCafferty (2003) made a systematic revision of the genus *Ephemerella* that collapsed many branches of the previous phylogeny by merging species with similar morphologies. For

example, 8 species including *E. inconstans* Traver, *E. floripara* McCafferty, and *E.rotunda* Morgan, were synonymized with the widely distributed species *Ephemerella invaria* (Walker). However, I and other ecologists working with these taxa maintained that morphologies of local populations of synonymized species are distinct and that species status is supported by differences in distribution, habitat, behavior, and life cycle (D. Funk, D. Lenat, personal communication).

The choice of DNA markers is one of many technical decisions to be made in a phylogenetic analysis. The decision to sequence a portion of subunit I of the mitochondrial cytochrome oxidase gene (mtDNA COI) for this phylogeny was based on the work of Simon *et al.* (1994) and on the fact that COI sequences from twelve *Ephemerella* populations were already available from a 2001 survey of mayflies in headwater streams (L. Alexander, unpublished data). The limitations of mtDNA and the COI gene in particular for determining evolutionary relationships has been thoroughly reviewed (Avise 2004, Neigel & Avise 1986), recently by Rubinoff *et al.* (2006) in response to the increasingly common use of this gene sequence for DNA-based species identification, a practice described by Hebert *et al.* (2003) as "DNA barcoding". The controversy surrounding the barcoding approach to taxonomy and as a tool for investigating global biodiversity has been discussed elsewhere (e.g., Moritz & Cicero 2004, Will & Rubinoff 2004). Specific practical issues relating to reconstruction of the evolutionary history of closely related species include the situation in which the coalescent has yet to sort between incipient species (ancestral polymorphism) so that intraspecific variation overlaps with interspecific divergence and gives rise to genetically polyphyletic or paraphyletic species (Meyer & Paulay 2005, Rosenberg 2003, Funk &

Omland 2003). When such overlap exists, the marker that is still in the process of sorting lineages cannot reliably distinguish among them. Test of the mtDNA COI gene as a barcoding tool for identifying *Ephemerella* species was not one of the initial objectives of this project, but was added when it became clear that the dataset I was developing could be used as an ad hoc evaluation of the utility of mtDNA as a taxonomic tool for rapid identification of mayfly species in this genus.

Mayflies comprise the taxonomic order Ephemeroptera, with 7 described families, 376 genera, and 3083 species that are distributed world-wide except the Arctic and Antarctica (Ogden & Whiting 2005). Ephemeroptera is the most basal extant lineage of winged insects (Grimaldi & Engel 2005) and is unique among present-day insects in having a subimago stage with fully functional wings (Edmunds & McCafferty 1988). The family Ephemerellidae, in the suborder Furcatergalia and infraorder Pannota, consists of two subfamilies, Ephemerellinae and Timpanoginae (McCafferty & Wang 2000), 20 genera, and over 300 species. The subfamily Ephemerellinae and genus *Ephemerella* Walsh (1862), the largest genus in Ephemerellidae, have undergone frequent revision in recent decades in North America (Allen & Edmunds 1962, Allen & Edmunds 1963, Allen & Edmunds 1965, Allen 1980, Allen 1984, Jacobus & McCafferty 2003). *Ephemerella* in particular has been notable for problems of paraphyly, poor diagnostics, and high population-level variability in some species, and was the focus of a recent revisionary contribution by Jacobus & McCafferty (2003). Ephemerellid mayflies have distributions and ecologies that are favorable to current research topics in stream ecology, including toxicology, nutrient transport and cycling, insect dispersal, stream re-colonization, and predator-prey interactions (Beketov 2004, Rezanka & Hershey 2003,

Benke & Jacobi 1994, McShaffrey & McCafferty 1991). Therefore, accurate mapping of species boundaries taxonomically and geographically is important to fields of research outside of systematics, as cryptic genetic diversity represents a source of uncontrolled experimental error in such studies.

To investigate relationships among *E. inconstans* and regionally disjunct populations of other *invaria* synonyms, I sequenced a short region of mitochondrial DNA (mtDNA) cyctochrome oxidase subunit I (COI) in samples across a large geographic range (~1400 km). Specifically, I designed this study to meet three objectives: 1) to estimate intraspecific genetic distances among synonyms and populations of *E. invaria* across the eastern range of the species; 2) to construct a molecular phylogeny of species closely related to *E. invaria;* and 3) to determine the utility of using short sequences of mtDNA to sort and identify morphologically ambiguous mayfly samples to species.

Materials and Methods

Taxon sampling

Samples (n=78) representing 12 species and synonyms were obtained from 13 geographic locations (Table 1a and Figure 1). Preserved tissue samples from specimens used in the systematic revision of the genus (Jacobus & McCafferty 2003) were obtained from L.M. Jacobus (Purdue University, Indiana). Additional tissue samples and voucher specimens were obtained from collections held at the Stroud Water Research Center (Avondale, PA) and at the North Carolina Division of Water Quality (Raleigh, NC).

Fresh samples were also collected in Maryland, Virginia, Pennsylvania, and North Carolina for this study. The fresh samples were collected in 100% ethanol, stored at

ambient temperature during transit and put into long-term storage at -20ºC. Heads were removed for DNA extraction and bodies (thoraces + abdomens) were labeled and stored as vouchers.

Three additional sequences were obtained from Genbank (Table 1b). Outgroup taxa and samples were provided by L.M. Jacobus (Table 1a).

Specimen processing

DNA was extracted using the DNEasy Kit and protocol (Qiagen, Chatsworth, CA). A small (380-490 base pairs) segment of the mitochondrial gene cytochrome oxidase subunit I (COI) was amplified by polymerase chain reaction (PCR) with primers 'Ron' (C1-J-1751, 5'-GGA TCA CCT GAT ATA GCA TTC CC-3', 23 bp) and 'Nancy' (C1-N-2191, 5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3', 26 bp) (Simon *et al.* 1994).

The cycling profile began with one cycle of DNA denaturation at 94°C for 2 min and followed by 35 cycles of sequence amplification (DNA denaturation at 94°C for 30 s, primer annealing at 47°C for 30 s and sequence extension at 72°C for 1 min). PCR products were treated with Exonuclease I (Exo) and Shrimp Alkaline Phosphatase (SAP) to degrade unincorporated primers and dNTPs. The sequencing reactions were carried out using ABI BigDye® v3.1 terminators and the resulting products were sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequencing reaction mixes contained 25 mol template, 1.25 pmol labeled primer, 2.75 mM MgCl2, 10 mM Tris-HCl, pH 9.2, 100 mM KCl, 0.01 U pyrophosphatase, and 1.4 µg Taq polymerase, 125 µM each dNTP and either ddATP, ddGTP, ddCTP or ddTTP at 1 µM in

a total volume of 20 µl. Thermal cycling consisted of 25 cycles of 10 s at 96°C, a 1°C/s ramp to 50°C, 15 s at 50°C, a 1°C/s ramp to 60°C, and 4 min at 60°C. Chromatographs of each sequence were examined to determine sequence quality, aligned using Sequencher (Gene Codes Inc., Ann Arbor, MI). Sequences obtained from forward and reverse primers were compared when needed to check base calls and confirm positions of polymorphic sites. The sequences were then edited in BioEdit (Hall 1999) to create nucleotide data matrices.

Phylogenetic analysis

Maximum parsimony and maximum likelihood analyses of the nucleotide matrix were conducted with PAUP* version 4.0b10 (Swofford 1998). Unweighted maximum parsimony analysis was done with heuristic searches using the tree bisection and reconnection (TBR) method of branch swapping (100 sequence-addition replicates). To assess the level of branch support, 1000 bootstrap replications were performed using the same search and optimization criteria except that the number of random sequence-addition replicates was reduced to 25. The maximum likelihood analysis used equally weighted trees from the parsimony analysis as starting points to estimate the log likelihood of trees obtained under a Hasegawa-Kishino-Yano+invariant+gamma (HKY+I+G) model of evolution (Hasegawa *et al.* 1985), with among-site rate variation modeled as a gamma distribution with 4 rate categories. The best-fit model (HKY+G + I; $\alpha = 0.8965$, $I = 0.5133$) was selected through a hierarchical likelihood ratio test on the Modeltest 3.07 software (Posada & Crandall 1998). As in the parsimony analysis, the TBR method of branch swapping was used. Maximum likelihood bootstrap analysis

(700 bootstrap replications) was conducted with GARLI (Zwickl 2006) version 0.951 with the model parameters from Modeltest.

Pairwise comparisons of the sequences were made and genetic distances within and among populations estimated using the Kimura 2-parameter (K2P) method in the software program DNADIST (PHYLIP, Felsenstein 1993).

Results

Maximum parsimony analysis

Of the sequenced base pairs, 137 (28%) were parsimony informative characters. Of these, 121 (88.3%) occurred in the third codon position; the other 16 were in the first codon position. Overall base frequencies were slightly biased towards A+T (59%), which is typical for insect mitochondrial genomes (Simon *et al.* 1994). A Chi-square test showed that base pair frequencies were homogeneous across taxa (p=1.0). In the parsimony analysis, 9 equally parsimonious trees of length 599 were obtained. Strict consensus of the 9 equally parsimonious trees (Figure 2) shows strong bootstrap support (92%) for grouping *E. inconstans* with *E. invaria,* but a second clade of haplotypes morphologically identified as *E. invaria* nested within the first clade also has strong bootstrap support (84%). The relationships of two other *E. invaria* synonyms, *E. rotunda* and *E. floripara,* are not well resolved by this analysis. In both trees, *E. floripara* is placed with *E. dorothea* (Figures 2 and 3), and although samples of *E. rotunda* from Pennsylvania and New York group with *E. invaria,* other samples from Maryland and Virginia do not. Specimens identified as *E. dorothea*, a well-known species about which there has been little if any recent dispute, grouped as two distinct genetic lineages (90% and 70% bootstrap support respectively) with overlapping geographic ranges. This

species was included in the present analysis because prior evidence identified it as the sister group to *E. inconstans* (Traver) (Sweeney *et al.* 1987, D. Funk unpublished data) and its cryptic diversity was a surprise. The synonmy of *E. infrequens* with *E. dorothea* (Jacobus & McCafferty 2003) is not supported by this analysis, as the sequences of *E. infrequens* from samples taken in Idaho do not group with either lineage of *E. dorothea.*

Likelihood analysis

The maximum likelihood tree (Figure 3) is concordant with the maximum parsimony tree (Figure 2) in branches with >50% bootstrap support.

Genetic distance analysis

Mean within-lineage K2P genetic distance is 2.9±0.7%; mean among-lineage genetic distance is 15.4±1.1%, where "lineage" is a clade or unresolved branch of the maximum parsimony strict consensus tree in Figure 2. In this analysis there is strong bootstrap support (92%) for the monophyly of *Ephemerella invaria* and *Ephemerella inconstans*. However, average K2P genetic distance among lineages in this clade is 5.5%, which is high relative to the average within-lineage genetic distance of 1.5% (Table 2). The nested clade labeled "INVARIA I", supported with a bootstrap value of 84%, has a geographic range extending from Maine to North Carolina (~1400 km) but average genetic distance of just 1.3% among sample sites (Maine, New York, Pennsylvania, Maryland, Virginia, and North Carolina). By contrast, samples of "INVARIA I" and samples of the lineage labeled "INCONSTANS" that were collected from the same stream reaches in Maryland and in Virginia had an average genetic distance of

4.7%, indicating that the differences observed here are not just the result of geographic distance. The specimens identified morphologically as *E. rotunda*, a new synonym of *E. invaria* (Jacobus & McCafferty 2003), comprise 2 genetic lineages labeled "ROTUNDA I" and "ROTUNDA II" that have diverged significantly (mean genetic distance=12.1%) and are not monophyletic with respect to other recognized species, including *E. dorothea* and *E. subvaria*. Additionally, large genetic divergences between 2 lineages of *E. dorothea* (12.9% mean K2P distance) and between the eastern and western populations of *E. excrucians* (18.7% mean K2P distance) were discovered. A plot of the frequency distribution of pairwise genetic distances between all individuals (Figure 4b) found considerable overlap of intra- and inter-specific variation.

Discussion

The strong lineages revealed here indicate that the current taxonomy underestimates the true diversity of the genus, even in apparently solid taxa like *E. dorothea* and *E. excrucians*. Paraphyly of lineages that may be in the process of diverging accompany morphological ambiguity and confound the attempt to diagnose and identify species. High levels of genetic divergence characterized many lineages in *Ephemerella*, but examples of divergences <2% over large geographic scales (1400 km) also were found, so the similarity within and divergence among lineages are not functions of the physical distance between sampled populations.

The present evidence from mtDNA supports the monophyly of an *invaria* clade, but suggests that the clade may not include *E. rotunda* or *E. floripara*. Evidence for synonomy of *E. inconstans* with *E. invaria* is strong, but the status of these divergent

lineages is open to question as the present paraphyly may be due to incomplete lineage sorting or hybridization. The sampled populations of *E. inconstans* in Maryland and Virginia clearly represent a single genetic lineage that has diverged from other members of the *invaria* clade by as much as 6.3%. This finding is consistent with my observation that in Maryland and Virginia *E. inconstans* emerges earlier than *E. invaria* and is found in relatively greater abundance in first-order streams (L. Alexander, unpublished data).

The difficulties such patterns of diversity present for diagnosing *Ephemerella* species, especially using a short segment of mtDNA as in the "DNA Barcoding" approach to species identification, may be present for other mayfly taxa as well. The risk of using a barcoding approach in such cases is that the method depends on full lineage sorting and the lack of any overlap between inter- and intra-specific variation (i.e. presence of the "Barcoding Gap", Figure 4a). The results presented here (Figure 4b) are consistent with the "alternative version of the world with significant overlap and no gap" described by Meyer & Paulay (2005) who, like Moritz & Cicero (2004), predicted that overlap of inter- and intra-specific variation would be greater when a larger proportion of closely-related taxa are included, especially in taxonomically understudied groups. This complicates the attempt to delineate discrete taxa, but illustrates the fact that species are composed of sets of independent, but interacting, genetic lineages with unique evolutionary histories and potentials. Recognizing this puts the emphasis back on comprehensive sampling of taxa for both morphologic and genetic diversity.

Table 1. Locality data, including (a) collection data for new specimens, and (b) accession numbers for Genbank sequences used.

a)

Species	Locality	Date	Collector	Label
*E. aurivillii**	MT: Sweet Grass Co, Sweet Grass Creek	10 June 2000	LM Jacobus	*MT aurivillii*
E. catawba	NC: Haywood Co, Big Creek	12 June 03	LM Jacobus	*NC catawba*
E. d. dorothea	TN: Blount Co, pond in Cades Cove	13-21 May 2001	LM Jacobus	*TN dorothea*
E. dorothea	PA: Chester Co., White Clay Creek	1 May 2005	D. Funk	*PA dorothea*
E. dorothea	MD: Appomattox Co., Fishpond Creek	_March 2001_	L.Alexander	*VA dorothea*
E. dorothea	MD: Howard Co., South Stream	_April 2003	L.Alexander	*MD dorothea 2*
E. dorothea	MD: Carroll Co., Morgan Run	20 March 2005	L.Alexander	*MD dorothea 1*
E. dorothea	NC: McDowell Co., Reedy Branch	21 April 2005	W. Crouch	*NC dorothea 1*
E. dorothea	NC: McDowell Co., Roses Creek	20 April 2005	W. Crouch	*NC dorothea 2*
*E. excrucians**	NE: Brown Co, Long Pine Creek	6 June 2000	LM Jacobus	*NE excrucians*
E. excrucians	FL: Okaloosa Co, Turkey Creek	12 April 2001	LM Jacobus	*FL excrucians*
E. floripara	NC: Caldwell Co, Wilson Crab Gorge	April 2003	D. Lenat	*NC floripara*
E. hispida	TN: Sevier Co., Dunn Creek	17 May 2001	LM Jacobus	*TN hispida*
E. hispida	NC: Transylvania Co., Big Bearpen Branch	22 April 2005	W. Crouch	*NC rossi*
E. inconstans	DE: Pratts Branch	11 April 1988	D. Funk	*DE inconstans*
E. inconstans	TN: Anderson Co, Clinch River tributary	21 May 2001	LM Jacobus	*TN inconstans*
E. inconstans	MD and VA: multiple sites	2001-2004	L.Alexander	*MD VA inconstans*
E. infrequens	ID: Valley Co., East Fork Salmon River	8-14 July 1989	D. Funk	*ID infrequens*
E. invaria	NY: Delaware Co., W. Delaware R.	13 May 2005	D. Funk	*NY invaria*
E. invaria	PA: Berks Co., Angelica Creek	6 April 2005	D. Funk	*PA invaria*
E. invaria	PA: Chester Co., White Clay Creek	5 April 2005	L.Alexander	*PA invaria2*
E. invaria	VA: Appomattox Co., Saunders Creek	12 April 2002	L.Alexander	*VA invaria*
E. invaria	MD: Carroll Co., Joe Branch	20 March 2005	L.Alexander	*MD invaria*
E. invaria	NC: McDowell Co., Buchanan Creek	20 April 2005	W. Crouch	*NC invaria 3*
E. invaria	NC: Caldwell Co., Wilson Crab Gorge	April 2003	D. Lenat	*NC invaria2*
E. invaria	NC: Caldwell Co., Wilson Creek	14 April 2005	D. Lenat	*NC invaria1*
E. rotunda	PA: Berks Co., Manatawny Creek	6 April 2005	D. Funk	*PA rotunda*
E. rotunda	NY: Delaware Co., W. Delaware R.	13 May 2005	D. Funk	*NY rotunda*
E. rotunda	VA: Giles Co, North of Pembroke Jeff. NF	11 March 2002	LM Jacobus	*VA rotunda*
E. rotunda	MD: Frederick Co., Fishing Creek	19 March 2001	L.Alexander	*MD rotunda*
E. subvaria	PA: Chester Co., White Clay Creek	5 April 2005	L.Alexander	*PA subvaria*
NC species a	NC: Richmond Co., Naked Creek	April 2005	D. Lenat	*NC sp a*
E.rossi	NC: NC: Transylvania Co., Bear Wallow Bk	21 April 2005	W. Crouch	*NC rossi*

* Outgroup taxon

b)

Species	GenBank accession number	Label
E. dorothea	AY326813	*WV dorothea*
E. invaria	AY326814	*ME invaria*
E. subvaria	AY326815	*Ontar subvaria*

Table 2: Genetic distances within and among lineages in the *E. invaria* clade. Values on the diagonal are distances within lineages; distances below diagonal are pairwise distances among lineages in the *invaria* clade. Mean within-lineage distance is 1.33±0.44%. Mean distance among lineages is 5.22±0.25%.

#	Branch name	1	2	3	4	5
1	INVARIA I	1.3				
2	ROTUNDA I	4.1	0.4			
3	INCONSTANS	5.1	5.4	2.3		
4	NC *invaria1*	5.3	5.3	6.5	1.3	
5	TN *inconstans*	4.7	4.0	5.7	6.1	--

Table 3: Genetic distances (K2P) within and among all taxa. Values on the diagonal are average distances within taxa; distances below diagonal are pairwise distances among taxa. Mean within-taxon distance is 2.9±0.69%. Mean distance among taxa is 15.4±1.1%.

	Branch name	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
1	*E.invaria* clade	4.4									
2	DOROTHEA I	13.4	6.4								
3	DOROTHEA II	16.0	12.9	7.0							
4	ROTUNDA II	13.4	11.8	14.1	2.6						
5	*E. hispida*	12.6	12.2	14.6	12.2	0.5					
6	*E. subvaria*	13.3	15.7	14.8	16.0	14.6	2.4				
7	NC *catawba*	11.4	14.9	15.7	13.0	13.4	15.1	--			
8	NC *floripara*	11.2	13.3	12.9	11.8	12.4	16.6	14.4	--		
9	FL *excrucians*	15.3	19.2	18.2	17.3	15.4	17.1	15.4	15.5	3.8	
10	NE *excrucians*	19.3	20.9	17.6	16.9	20.5	20.4	15.4	17.0	18.6	--
11	MT *aurivillii*	32.7	34.1	34.3	33.3	32.4	37.0	29.7	32.7	18.6	29.5

Figure Captions

Figure 1. Map of ingroup sample sites.

Figure 2. Strict consensus of 9 equally parsimonious trees.

Figure 3. Maximum likelihood tree based on HKY+I+G model of evolution.

Figure 4. Frequency distribution of pairwise genetic distance estimates.

(a) The DNA barcoding concept predicts that intraspecific and interspecific genetic distances will be distributed bimodally, with a gap between conspecifics and congenerics; (b) The actual distribution in *Ephemerella*. Considerable overlap between conspecifics and congenerics fills the "barcoding gap".

Figure 1. Map of ingroup sample sites.

Figure 2. Strict consensus of 9 equally parsimonious trees. Values shown are bootstrap support (1000 bootstrap replicates). Branch names (SMALL CAPS) denote lineages within species.

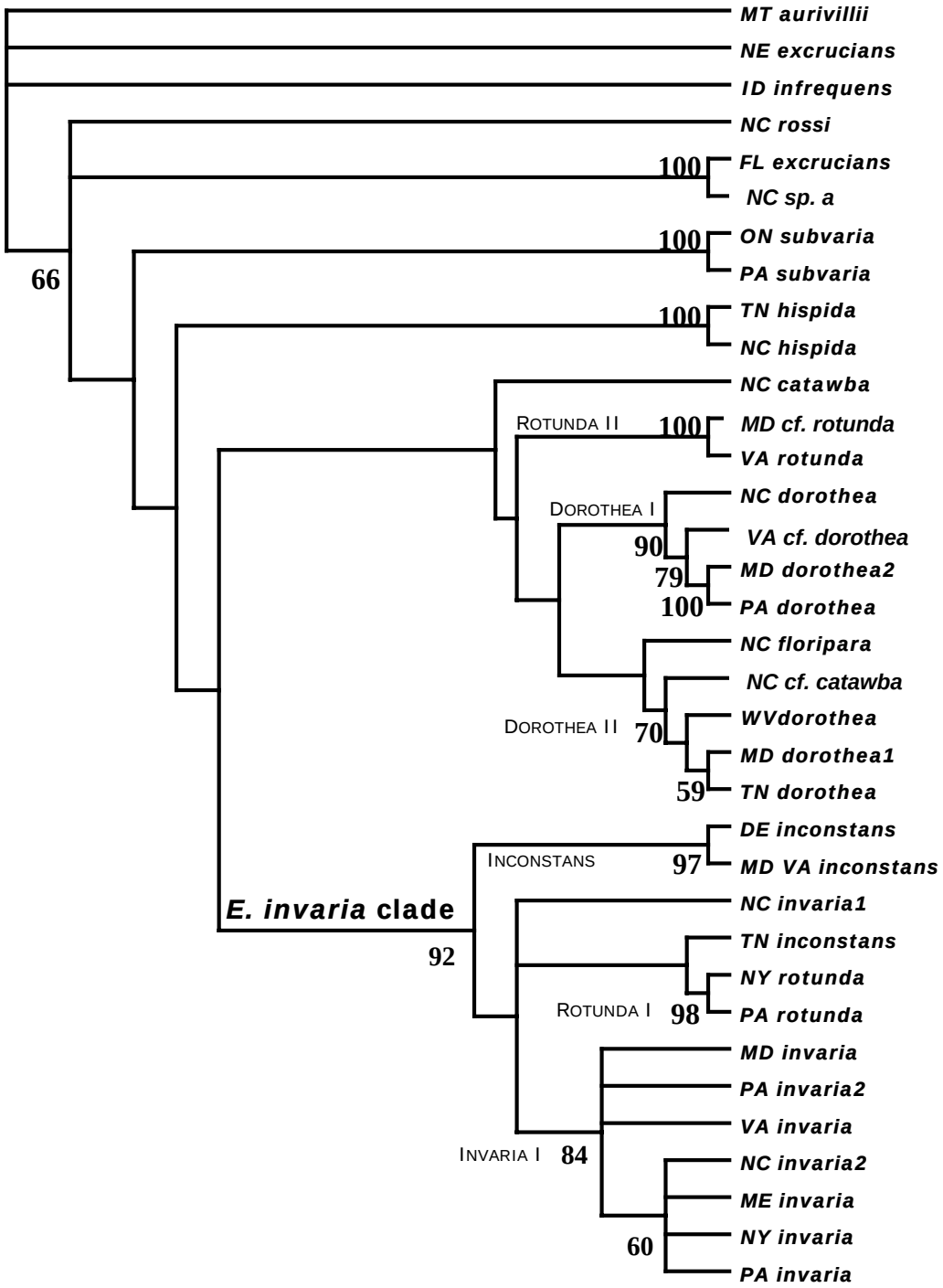


Figure 3. Maximum likelihood tree based on HKY+I+G model of evolution.

Values shown are bootstrap support (700 bootstrap replicates).

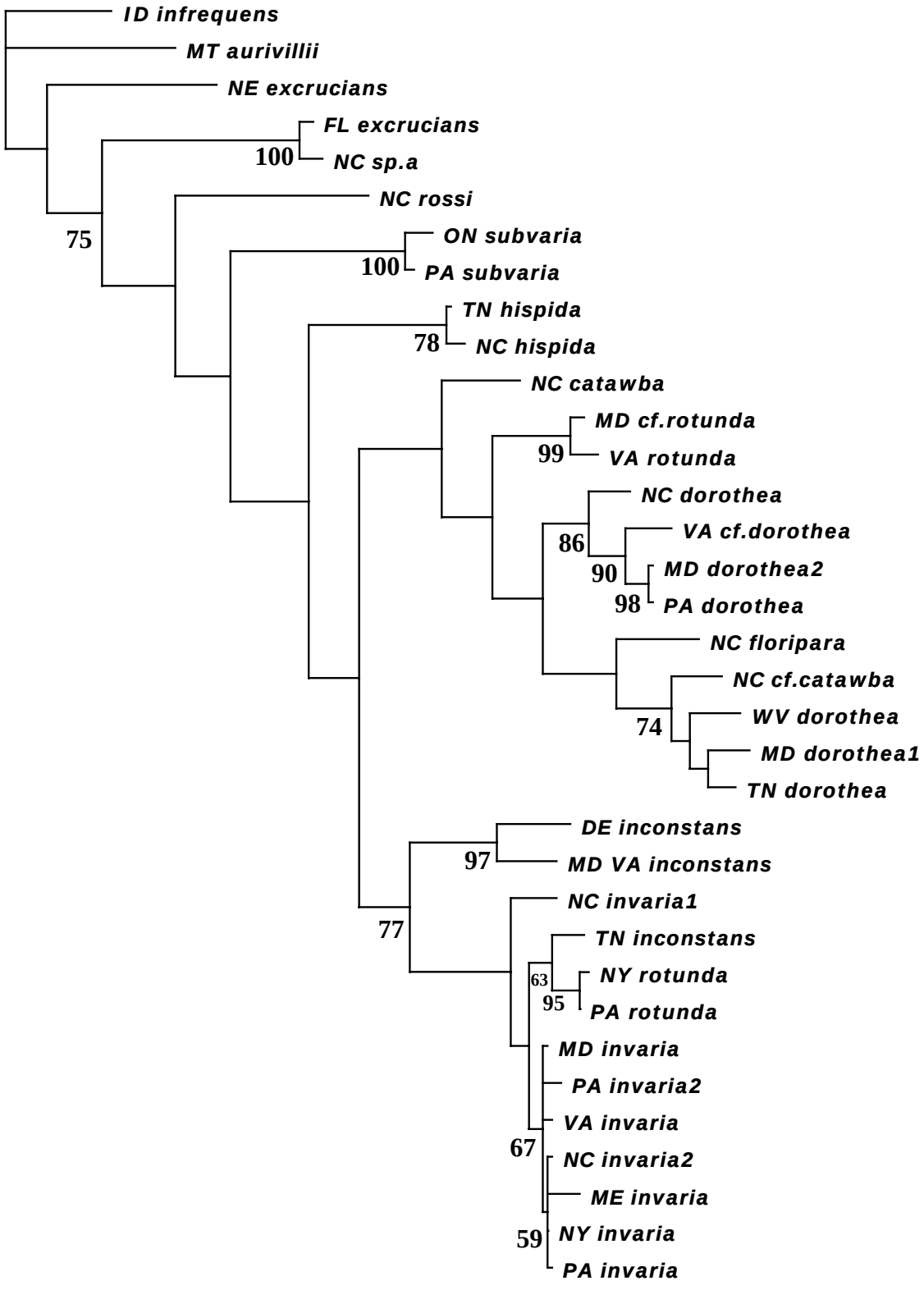
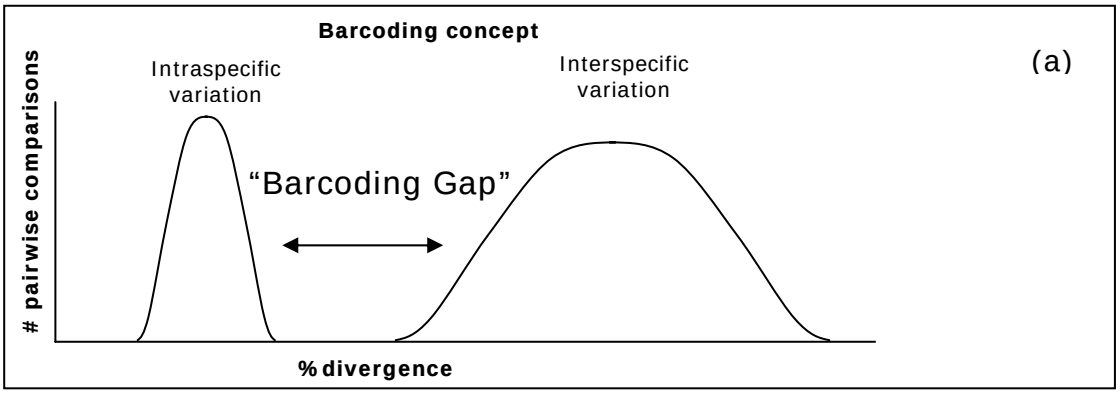
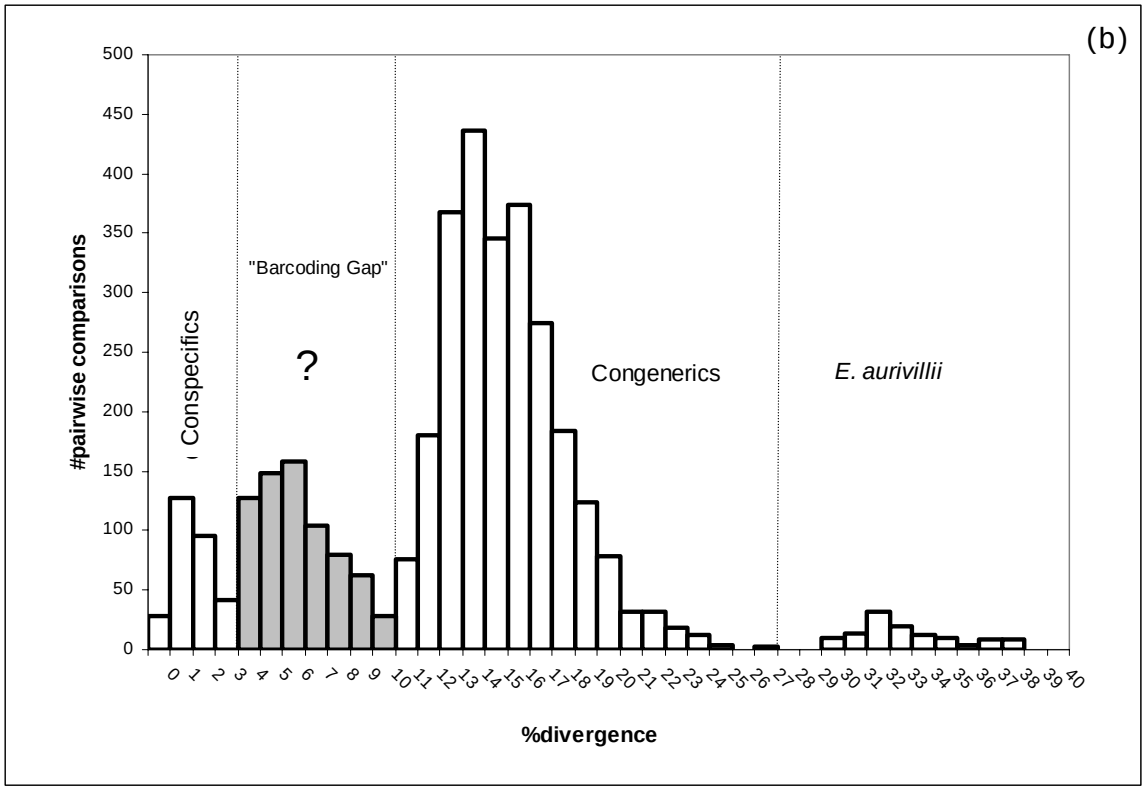


Figure 4. Frequency distribution of pairwise genetic distance estimates.
(a) The DNA barcoding concept predicts that intraspecific and interspecific genetic distances will be distributed bimodally, with a gap between conspecifics and congenerics (b) The actual distribution in *Ephemerella*. Considerable overlap between conspecifics and congenerics fills the "barcoding gap".





CHAPTER II: Population Genetic Diversity of an Ephemerellid Mayfly in Deforested Headwater Streams

Abstract

I assessed the effects of deforestation on the population genetic structure of a mayfly (*Ephemerella invaria*, Ephemerellidae) in 14 first-order streams across 9 headwater stream networks in Maryland and Virginia. Under a model of dispersal in which populations in semi-isolated headwater streams are connected primarily through aerial migration, I predicted that genetic diversity would be higher and the level of population structure lower in forested stream sites compared with deforested (agricultural and residential) sites. Using amplified fragment length polymorphism (AFLP) markers, I found high (θ_B= 0.18 - 0.20) population structure among sites in three regions over distances ranging from 100-300 km, but low (θ_B = 0.03) to moderate (θ_B = 0.06 – 0.10) population structure between sites within and among major river watersheds (2-100 km), indicating that these mayflies are capable of dispersing beyond their natal first-order watershed. Additional evidence of long-range dispersal was an assignment test based on estimated allele frequencies at each AFLP locus in which a small number of individuals were strongly admixed across a distance of over 300 km. A Mantel test of correlation of pairwise population genetic and geographic distances was significant, indicating that a process of isolation-by-distance has occurred. A high degree of polymorphism was observed in all populations (average 64.5% polymorphism), despite evidence of a regional population bottleneck from a previous study using mitochondrial DNA markers. Genetic diversity was significantly higher in forested streams compared with streams flowing through agricultural and residential areas, and percent polymorphism, estimated

heterozygosity, and mean pairwise genetic distance within populations are all negatively correlated with the degree of deforestation. These results indicate that this mayfly is capable of cross-watershed dispersal and that historic polymorphisms accumulated in this species have been retained over many generations, even under conditions of population bottlenecks. However, deforestation of small watersheds may currently be adversely affecting population processes of ephemerellid mayflies living in them, even when a forested riparian buffer remains. I discuss some possible explanations for the loss of present-day genetic diversity in a species that has demonstrated its ability to retain diversity through difficult conditions in the past.

Introduction

Discontinuity introduced by habitat fragmentation alters the size, number, distribution, and genetic composition of populations. Taxa respond differently to habitat loss and fragmentation, but the general patterns that have emerged are reduction in demographic size, increased demographic stochasticity, reduction in levels of gene flow, and loss of genetic diversity (Ewers & Didham 2005, Luck *et al.* 2003, Ceballos & Ehrlich 2002, Hughes *et al.* 1997, Frankel & Soulé 1981). Predicting the impact of habitat loss on populations and species depends in part on understanding how individuals move among resource patches and population units (Turner *et al.* 2001, Goodwin & Fahrig 2002) and how differences in the matrix affect dispersal and movement of individuals in fragmented systems (Ricketts 2001, Davies *et al.* 2001, Moilanen & Hanski 1998, Bierregaard & Stouffer 1997, Gustafson & Gardner 1996).

Movements of individuals shape the spatial structure of populations and species, and play an important role in their persistence (Hanski & Ovaskeinen 2002, Lowe 2002). Some movements, such as annual migration, are predictable; others, such as catastrophic stream drift by insects, are responses to random events or local conditions (Humphries 2002, Ledger *et al.* 2002, Anholt 1995). Direct observation is the most reliable source of information about insect dispersal, but is impractical at large scales. Consequently, recent efforts have focused on improving methods for inferring movement from indirect data (mark-recapture or genetic samples), drawing from theoretical population biology, population genetics, and landscape ecology (Turchin 1998). Methods in landscape genetics, which integrate these disciplines to understand how geographical and environmental factors structure genetic variation at the population and individual levels (Manel *et al.* 2003), have been successfully applied to current questions about the status of freshwater resources that support invertebrate populations (Hughes *et al.* 2003, Schultheis *et al.* 2002, Monaghan *et al.* 2001, Myers *et al.* 2001).

The focus of my research has been on effects of land use on populations of aquatic insects living in the small streams that form the origins of larger river networks. Small streams are naturally patchy habitats that support diverse communities of aquatic insects (Meyer *et al.* 2007). Individual headwater streams may be only a few hundred meters in length and flow through watersheds less than one square kilometer in area (Leopold 1997). In addition to providing habitat and refuge, headwater streams and their associated wetlands perform ecological functions of importance to the larger ecosystem, including slowing runoff, retaining sediment, recharging groundwater sources, taking up chemicals and excess nutrients that would otherwise be transported to bays, lakes, and

oceans, and processing organic matter (Peterson *et al.* 2001a, Progar *et al.* 2002, Wallace *et al.* 1997, Lowe & Likens 2005). The disproportionately large role that small streams play in ecosystems is possible in part to their distribution and abundance. Recent surveys estimate that headwater streams comprise a minimum of 80% of stream miles in the United States (Meyer *et al.* 2003) and at least 66% of stream miles in Maryland (Maryland Department of Natural Resources 1997, 2001) where they are distributed as complex networks covering large areas over which flow is diffused through many small channels.

Extensive deforestation of small watersheds in the Mid-Atlantic Piedmont region of North America has altered the structure and function of headwater streams by reducing their number, disrupting ecosystem processes, and fragmenting surviving headwaters into isolated or semi-isolated habitat patches (Maryland Department of Natural Resources 1997, 2001). Most deforestation in Maryland and Virginia occurred tens or hundreds of years ago as land was cleared of trees for agriculture (Foresman 2003, White & Mladenoff 1994, Riiters *et al.* 2002). Although some tracts have been re-forested, especially in state and national parks, past land use continues to influence stream biodiversity (Harding *et al.* 1998), and human population growth and urbanization continue to alter hydrologic and hydrobiologic conditions across the region (Beighley & Moglen 2002, Moglen 2000). The shift in land use from forested to agricultural to residential and urban has reduced community-level diversity in stream invertebrates (Moore & Palmer 2005) and altered stream ecosystem functioning (Brooks *et al.* 2002, Meyer *et al.* 2005). In recent years, efforts in Maryland and elsewhere have made progress in the restoration of stream habitats, emphasizing the establishment and

protection of stream buffers and riparian vegetation and the restoration of ecosystem function (Palmer *et al.* 2002, Moglen 2000, Hassett *et al.* 2005, Palmer *et al.* 2005).

Because the preservation of natural connections for movement of individuals among semi-isolated populations in geographically structured habitats can significantly affect the extinction probabilities of those populations (Fagan 2002, Hanski & Ovaskeinen 2002, Lowe 2002), threats to population persistence must be evaluated in a regional context with emphasis on understanding new rules imposed on populations by changing landscape structure. Fagan (2002) and Fagan *et al.* (2002) found that dendritic habitat structure increases fragmentation effects with negative consequences for population persistence in desert fishes. Historically in the central piedmont of North America, streams channels were naturally hierarchical and dendritic, but forests were broadly two-dimensional. Today, surviving and restored forest patches follow stream corridors, so the new distribution of forests in this region is also dendritic. The re-arrangement of trees in a watershed could interact with the dispersal abilities and behaviors of stream insects with aerial adult stages, imposing new constraints on movement that could alter patterns of population distribution and abundance (Grant *et al.* 2007).

I assessed the effects of deforestation on the genetic diversity and population genetic structure of an ephemerellid mayfly (*Ephemerella invaria*) in 14 forested and deforested first-order streams across 9 headwater stream networks in Maryland and Virginia using Amplified Fragment Length Polymorphism (AFLP) markers. My objectives were two-fold: to determine how population genetic structure and diversity varied with the degree of watershed deforestation; and to infer from that relationship the

properties of terrestrial connectivity among headwater stream habitats needed to maintain regional genetic diversity in populations of *Ephemerella invaria*. Under a model of dispersal in which mayfly populations in semi-isolated headwater streams are connected primarily through aerial migration, I predicted that population genetic structure would be lower, and genetic diversity higher, in forested stream sites compared with deforested (agricultural and residential) sites (Bohonak 1999, Hartl & Clark 1997, Felsenstein 1982), where genetic distance among populations would be significantly related to geographic distance (isolation-by-distance) even at small scales (<10 km). I also predicted that the combined effects of reduced population size and connectivity would result in negative correlation of deforestation to genetic diversity, at local and regional scales (Pannel 2003, Pannel & Charlesworth 1999).

Study System

Mayflies comprise the taxonomic order Ephemeroptera, with 7 described families, 376 genera, and 3083 species that are distributed world-wide except the Arctic and Antarctica (Ogden & Whiting 2005). Ephemeroptera is the most basal extant lineage of winged insects (Grimaldi & Engel 2005) and is unique among present-day insects in having a subimago stage with fully functional wings (Edmunds & McCafferty 1988). The family Ephemerellidae, in the suborder Furcatergalia and infraorder Pannota, consists of two subfamilies, Ephemerellinae and Timpanoginae (McCafferty & Wang 2000), 20 genera, and over 300 species. The subfamily Ephemerellinae and genus *Ephemerella* Walsh (1862), the largest genus in Ephemerellidae, have undergone frequent revision in recent decades in North America (Edmunds 1959, Allen 1965, Allen

& Edmunds 1965, Allen & Edmunds 1968, Allen 1980, Allen 1984), most recently by Jacobus & MacCafferty (2003) who synonymized multiple species based on morphology. Chapter 1 of this dissertation followed with a molecular analysis of genetic lineages in *Ephemerella invaria* and its recently synonomized congeners. Based on these contributions I am confident that specimens in the current study belong to a single genetic lineage within the species *E. invaria*. Populations of *E. invaria* are highly variable in morphology, habitat, distribution, and genetic composition, so to avoid confusion I will refer to this particular lineage of *E. invaria* by the name *Inconstans*.

Biology

In the Mid-Atlantic Piedmont region populations of the *Inconstans* lineage are abundant in headwater streams with variable in-stream habitat structure and water quality. A survey of 25 first-to-third order streams in Maryland found the *Inconstans* lineage of *E. invaria* broadly distributed in headwater streams across the region, except in areas with heavy urban or residential land use (L. Alexander, unpublished data).

The life cycle is univoltine, with emergence in Maryland starting during the second or third week of April, peaking in early May, and concluding in the third or fourth week of May. Nymphs are typically large (11-13 mm) and compared with other local *Ephemerella* species, slow-moving. Emerging nymphs swim to the stream surface and drift momentarily before molting. Once initiated, eclosure is rapid and subimago flight occurs almost immediately after molting. Subimagos fly straight up from the stream surface, ascending out of sight to high branches in riparian trees. In laboratory conditions the subimagal and imagal stages ranged from 24 to 36 hours each, for a total winged

stage of 48 to 72 hours. To my knowledge mating swarms have never been observed in this lineage, but ovipositing females have been seen descending 12 or more meters above the stream surface where high branches of riparian trees hang over the stream. Females descend within a few centimeters of the stream surface, often touching the abdomen to the surface of the water, and drop their egg masses into stream riffles. Descending females may fly up or downstream before ovipositing. Eggs masses contain 300-500 eggs and sink quickly (i.e. with little downstream drift) where they separate and adhere to the substrate within 30 minutes. Eggs diapause through the summer. Although it is not known precisely when the eggs hatch, early instar nymphs may be collected in Maryland starting in September. The number of instars for this species is unknown. Early instars live in the substrate or in vegetation at the stream margins. Later instars (>3) are common in root wads or other complex vegetation at the stream margins, although in rapid, silt-free flow they may be abundant in gravel substrates. Their legs are adapted for clinging, and they hold tenaciously to stringy substrates. They feed by grazing on the surface biofilm on the substrate (e.g., root strands). Complex feeding behavior on filamentous algae (*Cladophora*) has been documented for another species in this genus (McShaffrey 1992) but the preferences of *E. invaria*, and the nutritional value of its available food sources, have not been studied.

Ephemerella nymphs and adults are prey for a variety of predators. The most common aquatic predators in Piedmont headwaters are stoneflies (e.g., Perlidae), caddisflies (e.g., Rhyacophilidae), amphibians (e.g., salamanders and frogs), and small fish (e.g., McPeek & Peckarsky 1998). Terrestrial predators, including birds, spiders, and bats, also exist but predation effects of this group have not been well studied.

The extent of dispersal is not known either. Nymphs in the *Inconstans* lineage have been collected in drift samplers in large numbers (L. Alexander, unpublished data) but upstream movement has not been documented. Aerial dispersal is possible in the winged stage, but the short duration of the winged life span makes mark-and-recapture studies impractical. Mayflies are often described as "poor fliers" (e.g., Edmunds *et al.* 1976), and although hard evidence of this assumed limitation is lacking, dispersal studies using Malaise traps typically find mayfly adults flying only within a few meters of the stream channel (e.g., Petersen *et al.* 2004). Mayflies are however clearly capable of rapid flight and precise maneuvering during aerial mating (Sartori *et al.* 1992), and Hughes *et al.* (2000) found indirect evidence of longer range dispersal in a genetic study of mayfly population structure.

Materials and Methods

Study Sites

I selected 9 headwater stream networks, each consisting of 1-4 first-order streams (study sites) with populations of *Inconstans* mayflies, located in 5 major river watersheds in Maryland and Virginia (Table 1 and Figure 1) for the study. Potential study sites were first selected by location and stream order using USGS 7.5 minute quadrangle maps. Partially or entirely forested sites in the Maryland or Virginia piedmont that contained at least 3 adjacent first order streams were identified. Watershed size, land use at the selected sites, and distance among sites were then analyzed with GIS digital elevation models (DEM) at a resolution of 30 meters/pixel using GIS Hydro 2000 (GIS Hydro 2000) for the Maryland sites and ArcView 3.3 (ESRI Inc., Redlands, CA.). Avenue

scripts based on GISHydro 2000 were developed for the Virginia sites. Non-urban watersheds no larger than 3 km^2 in area that had at least 50% of the first-order watershed area categorized as Forested, Agricultural, or Residential land use were surveyed visually to assess riparian land use, stream channel condition, and accessibility. At least 4 sites within each land use category (Forested, Agricultural, Residential) were selected for quantitative invertebrate sampling using the methods described below. Samples from all sites were sorted and *Ephemerella* mayflies identified to species (Allen & Edmunds 1965). If no *E. invaria* nymphs were ever found at a stream or site (i.e. no evidence of populations having been established in recent years), it was dropped from the study. Replacement streams/sites were added when possible, using the same selection process. The final number of headwater stream networks was reduced then to nine (9), with a total of fourteen (14) streams that had a range of GIS-estimated forest cover. Forest land use and land cover estimates from the GIS were visually assessed in all watersheds and found to be consistent with GIS estimates. GIS-estimated forest cover was ground-truthed using a handheld GPS in one watershed and found to be accurate.

Sampling

In 2001, 2002, and 2004, all nymph samples were collected using moss-packs (colonizing samplers) consisting of 2.5 g dried moss enclosed in plastic mesh bags and tied with string to roots or stakes along the stream margin for a period of 3 weeks in March and April, when late instar nymphs are present in the stream margins. Moss-packs are designed to move freely with streamflow to imitate natural moss or root-wad habitats. They are readily colonized by *E. invaria* and other aquatic invertebrate taxa. Eight moss-

packs were placed in each stream, positioned in pairs along a 75 m reach so that a total of 4 sub-samples were taken in each stream. The pairing of moss-packs was done to provide redundancy in case one moss-pack was buried, lost or moved out of the flow. The actual location of a moss-pack pair was selected at random and recorded with a GPS waypoint and hand-drawn map.

Samples were bagged in stream water and processed alive. Mayfly specimens were separated by species, placed in 100% ethyl alcohol, and stored at –20°C. When a stream sample contained fewer than 16 individuals total, that stream was re-sampled with a D-frame net, in an attempt to increase the size of the sample available for population genetic analysis. These samples were labeled as D-frame samples and stored separately from the moss-pack samples.

In 2003 nymph samples were collected with a D-frame net. A starting point along the stream was selected at random to define the start of a 150 meter reach, divided into 6 sections 25 meters in length. Of these sections, 3 were selected at random for sampling. All habitats suitable for *E. invaria* were sampled exhaustively within the 3 randomly selected sections. Moss pack samples were also taken in one stream for comparison with the D-frame samples.

Specimen collection and preservation

Fresh samples were collected into 100% ethanol, stored at ambient temperature during transit and put into long-term storage -20ºC. Heads were removed for DNA extraction and bodies (thoraces + abdomens) were labeled and stored as vouchers. Prior to extraction, the heads were frozen in liquid nitrogen and pulverized. DNA was

extracted from a total of 208 individuals representing 14 populations from 9 geographic locations (Table 1 and Figure 1) using the DNEasy Kit and protocol (Qiagen, Chatsworth, CA).

Amplified Fragment Length Polymorphism (AFLP)

The AFLP method (Vos *et al.* 1995) provides an unbiased estimate of whole-genome variation and repeatable, high-resolution differentiation of genetically related populations. The method works by digestion of genomic DNA with a pair of restriction enzymes, ligation of double-stranded adapters to the ends of the restriction fragments, amplification of the modified fragments with adapter-specific primers in 2 rounds of PCR, and visualization of the PCR product with gel or capillary electrophoresis. After a sample is processed, the resulting bands or peaks are scored for presence or absence and the binary pattern thus produced identifies the genotype of that individual. The AFLP procedure requires no primer design per se, because PCR primers are specific to the sequences of the recognition sites and ligated universal adapters. The choice of primers is made by testing different primer pair combinations to find pairs that generate adequate numbers of bands or peaks for estimating genetic polymorphism without producing so large a number that the results cannot be interpreted.

In an AFLP analysis, each band or peak represents presence of a restriction site at a particular locus and of the internal 1, 2, or 3 bp of flanking sequence. Therefore this technique actually measures polymorphism in and near the restriction site sequence, not restriction fragment length, and cannot differentiate between heterozygotes and dominant homozygotes. Using dominant markers to estimate population heterozygosity, an

informative measure of genetic diversity, can be problematic. Traditional methods based on co-dominant marker data require prior knowledge of the population inbreeding coefficient (F_{IS}) or the assumption of Hardy–Weinberg equilibrium (HWE), and can produce biased estimates (Lynch & Milligan 1994, Holsinger *et al.* 2002). Bias is less severe for estimates of population parameters obtained from dominant markers when a large number of polymorphic loci are used (Krauss 2000), as is often the case in AFLP and RAPD studies of natural populations. Alternatively, Bayesian analyses developed by Holsinger *et al.* (2002) and implemented in the software package Hickory (Holsinger 1999), provide nearly unbiased estimates of population genetic structure and heterozygosity without prior decisions about the inbreeding coefficient or Hardy-Weinburg equilibrium. Under appropriate conditions, this approach also estimates the inbreeding coefficient (F_{IS}), under the assumption that the level of inbreeding is the same for all loci.

AFLP fragment construction and amplification

AFLP fragments for PCR were constructed by mixing 10 uL genomic DNA, Fermentas Buffer "O" (Fermentas, Burlington, ON), 10 mM ATP, 10 units PstI enzyme, 10 units EcoRI enzyme, 2 units T4 DNA ligase, and 5 pmols of each double-stranded adapter (Table 2). The mixture was incubated overnight at 37ºC in a shaker oven. The restriction and ligation steps of the AFLP reaction were performed simultaneously in a total reaction volume of 25 uL. The first amplification reaction (preselective amplification) was run with primers that are complementary to the adapter sequence only (Table 2). The second amplification (selective amplification) was done using primers

that had 2 or 3 overhanging nucleotides at the 3' end (Table 2). Both reactions were run in a standard PCR cocktail [20 mm Tris-HCl (pH 8.4), 50 mm KCl, 1.5 mm MgCl2, 0.2 mm dNTPs, 1 unit Taq DNA polymerase] that included 5 pmols of each primer (Table 2). The preamplification reaction contained 5 uL of the AFLP construct (diluted 1:2 with ultrapure water) as template and was cycled 20 times for 1 min at 94ºC, 1 min at 56ºC, and 1.5 min at 72ºC. The selective amplification contained 2 uL of 10:1 diluted preamplification product as template and was run as a touchdown-PCR in which the annealing temperature was high (65ºC) for the first round and then reduced 0.7C for each of the next 12 cycles. The denaturing and extension stages for each cycle were 94ºC for 10 sec and 72ºC for 90 sec, respectively. This ramping-down of the annealing temperature was followed by 25 cycles of 94ºC for 10 sec, 56ºC for 40 + 1 sec per cycle, and 72ºC for 90 sec.

AFLP fragment detection and analysis

Fluorescent dye-labeled selective amplification products were diluted 10:1 with ultrapure water prior to capillary electrophoresis on an ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA). Electrophoresis mixtures consisted of 2 µl of 10:1 diluted PCR product and 8 uL of deionized formamide and X-Rhodamine labeled MapMarker®1000 size standard (BioVentures Inc., Murfreesboro, TN) in a 150:1 ratio. Data collection, processing, fragment sizing, and pattern analysis were done using the AFLP functions provided by ABI in GeneMapper version 3.7 software (Applied Biosystems, Foster City, CA), with factory default settings except as described here. Only fragments in the range from 50 to 600 bp were analyzed. Markers were selected by

initially setting the peak detection threshold to 50 relative fluorescence units (rfu) and the Genemapper 3.7 "allele-calling" threshold to 300 rfu. The project panel and bin set produced were then applied to the same set of individuals with the allele-calling threshold reset to 100 rfu for genotyping. To further reduce the number of spurious and low-frequency peaks, loci at which the presence of a fragment occurred in fewer than 5% of the total number samples were deleted from the data matrix and the final genotype table was converted to a character matrix for population analysis.

For gel-based AFLP, multiple primer pairs are used to generate an adequate sample of loci, as the number of unambiguous bands per gel/primer pair is typically low (<20). With capillary electrophoresis, the number of unambiguous AFLP fragments generated per primer pair is often an order of magnitude greater for natural populations with moderate to high genetic variation. In the present analysis, one primer pair produced a large number of loci for the population genetic analysis. However, to test for bias at those marker loci, I re-analyzed a random subset of individuals from 11 of the 14 populations using the second primer pair for a replicate sample of the genomic variation in those individuals. Estimates of population parameters from the separate datasets produced by each primer pair were correlated, and the average of both sets of parameter estimates were used in the analysis.

Population genetic structure and genetic diversity

The AFLP presence/absence matrix was analyzed with the software programs Hickory 1.0.5 (beta version) (Holsinger 1999, Holsinger *et al.* 2002), AFLP-SURV 1.0 (Vekemans 2002), and Arlequin 3.0 (Excoffier *et al.* 2005). Three measures of

population diversity from methods with different assumptions and implementations are reported. The first of three estimates of population diversity is the average panmictic heterzygosity within each population (*hs*) calculated in Hickory using the f-free model.

The second measure of population diversity reported is the proportion of polymorphic loci in each population sample, calculated by AFLP-SURV. The third diversity estimate reported here is within-population genetic distance, calculated as mean pairwise difference between individuals within each sample using the software program Arlequin v.3 (Excoffier *et al.* 2005).

Linear regression in SAS (SAS Institute, Cary, NC) was used to model the relationship between watershed deforestation and each of the measures of population diversity. "Percent deforestation" of each watershed is set to 1 – the percentage of forested land area.

A Mantel test (Mantel 1967) was conducted to determine if genetic distance is significantly associated with geographic distance for ten Maryland populations and three Virginia populations. IBD (Isolation By Distance) Web Service (Jensen *et al.* 2005, Bohonak 2002) was used from matrices of pairwise Fst values and Slatkin's (1993) $\hat{M}$ values calculated in Arlequin. The strength of the association was evaluated by reduced major axis (RMA) regression and confidence intervals calculated by jackknifing over population samples in the IBD Web Service.

To test for hierarchical population structure, populations were grouped by geographic location (major river watershed, geographic region) and estimates of θ_B and *Gst* were calculated using Hickory. Hickory implements Bayesian estimation with standard Monte Carlo Markov Chain (MCMC) methods to approximate the posterior

distributions of θ_B, a measure of population subdivision, and the inbreeding within populations, f. These statistics are analogous to Wright's (1931) F-statistics F_{st}, the correlation of gametes within subpopulations relative to gametes drawn at random from the entire population, and F_{is}, the correlation of uniting gametes relative to gametes drawn at random from within a subdivided population averaged over all subpopulations.

I also used the program Structure version 2.0 (Pritchard *et al.* 2000), a model-based (Bayesian) clustering method, to estimate the number of source populations from the samples of AFLP genotype data without prior information about geographic or populations structure. In Structure, individuals are assigned to populations characterized by allele frequencies at each locus. Under a model of admixture, one individual may be assigned to multiple populations; thus, Structure can be used to identify putative migrants or descendents of migrants in each population. I ran 3 iterations of the Structure analysis for K=2 to K=10 with 50,000 burn-in cycles and 100,000 MCMC replications each.

Results

Watersheds

The watershed characteristics are summarized in Table 4. Mean watershed area is 1.39 km^2. The degree of deforestation across the sample sites range from 1% (Gunpowder State Park, MD) to 83% (UMD Dairy Farm, Clarksville, MD). The matrix of Euclidean distances among the Maryland populations are given in the lower half of the isolation-by-distance matrix (Table 5).

AFLP genotypes

Genetic variation was high within and among populations. For both primer pairs, each individual generated a unique AFLP profile. On average, 64.8% of the analyzed loci were polymorphic within a population (Table 6).

One primer pair (Eag-Paca, Table 3) was used to genotype 208 individuals from 14 populations; a second primer pair (Eag-Paga, Table 3) was used to genotype 96 individuals from 11 of the 14 populations. The first primer pair (Eag-Paca) produced a total of 748 scorable loci of which 671 were polymorphic (90%). After eliminating polymorphic loci that occurred in fewer than 5% of the samples (as described above), a total of 471 loci were retained for analysis (Table 3). The second primer pair (Eag-Paga) produced a total of 545 scorable loci of which 424 were polymorphic (78%). Of these, 323 occurred in at least 5% of the samples and were thus retained for analysis (Table 3).

Correlation of population parameter estimates for 11 populations produced by each primer pair is shown in Figure 2. The population parameter estimates reported here are averages of estimates based on each primer pair.

Population genetic structure and diversity

The three estimates of genetic diversity within populations, and the number of polymorphic loci in each population, are summarized in Table 6. The relative diversity in each population was similar for each metric, and all measures of diversity were significantly correlated with the level of deforestation in the low-order watersheds (Figure 3). Values of θ_B and *Gst* estimated by Hickory for all populations, for

populations within regions, and populations within major river watersheds, are shown in Table 7.

Model choice for Bayesian parameter estimation

Model choice for the Bayesian estimation in Hickory followed the procedure introduced by Spiegelhalter *et al.* (2002), and advice in the software user manual written by Holsinger & Lewis (2003). I ran four models: (1) the full model, in which the coefficients of population structure and inbreeding are assumed to be unknown and may be other than 0 ($\theta \neq 0$ and $f \neq 0$); (2) the f=0 model, in which HWE is assumed and only the population structure coefficient is estimated ($f = 0$ and $\theta \neq 0$); (3) the theta=0 model, in which population structure is assumed to be absent and only the inbreeding coefficient is estimated ($\theta = 0$, $f \neq 0$); and (4) the f-free model, in which values of the inbreeding coefficient f are chosen at random from the prior distribution (uniform [0,1]). Using Spiegelhalter's Deviance Information Criterion (DIC) statistics, the full model was preferred (DIC=10990.7 versus DIC=11019.7 for f=0 and DIC=16800.2 for θ=0). The DIC takes into account the fit of the model to the data as well as the number of parameters estimated. The relative contribution of each is output by Hickory as Dbar (a measure of model fit to data) and pD (the number of parameters estimated). Holsinger & Wallace (2004) recommend using Dbar and pD as well as DIC in model choice, since it is possible that a model with poorer fit has a lower DIC (i.e., is preferred) simply because it required estimation of fewer parameters. In this case the DIC and pD were both minimized in the full model, so on this basis the full model would be preferred. However, the inbreeding level estimated with the full model was nearly equal to one

(f>0.98). If true, this value of f would mean that the populations in this study are almost completely inbred. This is inconsistent with the high level of polymorphism found in the AFLP markers, the high within-population heterozygosity estimated by all models including the f-free model, and the available facts regarding the mating system and natural history of the study populations. In the user's manual, Hickory's authors note that inbreeding is only weakly identifiable from dominant markers and advise users to treat the software's estimation of f with caution, especially if the estimate from the software is inconsistent with information from other sources, or based on small samples or a large number of loci. For this reason, I report the results from the f-free model (Table 7) which chooses values of f at random from the prior distribution (uniform [0,1]), thus incorporating all of the uncertainty about the level of inbreeding in the population into the estimate of population structure (θ_B) and heterozygosity (hs).

Isolation by distance

The Mantel test found a significant association of population genetic distance and geographic distance (r = -0.7612, p< 0.0001). A log-log regression of gene flow ($\hat{M}$) over geographic distance is plotted in Figure 4 (R^2=0.58, log($\hat{M}$)=1.209 – 0.5693 log($distance_{km}$)). The negative slope and fan-shaped pattern in Figure 4 are typical of IBD, as the homogenizing influence of gene flow predominates among closely located populations and the diversifying influence of drift and selection takes over as geographic distances increase. In Figure 4, streams located within 10 km have the most variable levels of population structure, with populations in upper left quadrant apparently experiencing higher levels of gene flow than those in the lower left quadrant.

Assignment Tests

Structure 2.0 identified two source populations, one in Maryland and the other in Virginia, with limited admixing in most individuals (Figure 6). The proportion of Maryland ancestry in the Virginia populations (Figure 6, red sections labeled a-e) ranged from 0.012 to 0.023. The proportion of Virginia ancestry assigned to the Maryland populations (Figure 6, green and predominantly green bars) ranged from 0.011 to 0.412. A small number of individuals in Maryland produced a strong signal of the Virginia allele frequencies indicating possible migrant individuals. These individuals are labeled with an asterisk (*) in Figure 6.

Discussion

The mayfly taxon *E. invaria* represents a genetically diverse species composed of distinct, but probably interbreeding, genetic lineages distributed over large geographic ranges in North America. Members of the *Inconstans* lineage, defined by the mitochondrial haplotype of early emerging populations that dominate the headwater streams of Maryland and Virginia in late winter and early spring, is an active disperser that has colonized small piedmont streams in large numbers. Populations of *Inconstans* mayflies exhibit a high level of nuclear DNA polymorphism accumulated over many generations, which it has retained even through conditions of population bottleneck as founders moved north following the end of the last Ice Age, and through subsequent smaller contractions as population segments colonizing different river watersheds diverged and differentiated.

However, modern deforestation of small watersheds may be adversely affecting population processes of *Ephemerella* mayflies, even in watersheds with forested stream buffers. I discuss some possible explanations for the loss of present-day genetic diversity in a species that has demonstrated its ability to retain diversity though difficult conditions in the past.

Long-term, long-range migration by *Ephemerella* mayflies is evident from the fact that all of the sampled headwater streams in the ~300 km range of this study have been colonized by members of a single mitochondrial DNA (mtDNA) lineage, and from the presence of admixed individuals in Northern Maryland with proportionally high levels of nuclear ancestry from the central Virginia gene pool. Low mitochondrial variation in invertebrates is not uncommon in the literature (e.g., Peterson *et al.* 2001b). Lack of concordance between patterns of diversity in mtDNA and nuclear DNA is usually attributed to differences in dispersal by males and females but could also be the result of unequal sex ratios, genetic drift, or balancing selection (Avise 2004, Moritz 1994). Due to the smaller effective population size of mtDNA, differences due to drift would likely produce stronger population structure in the mitochondrial DNA markers than in nuclear DNA markers, the opposite pattern from that observed here. Because the effective population size of mtDNA is one quarter that of nuclear DNA, mitochondrial polymorphism would be 4x more sensitive to the purifying effects of a population bottleneck. There is no evidence of unequal sex ratios in the study populations, but data on sex-biased dispersal is lacking.

The current large-scale pattern of population structure for *Ephemerella invaria* is consistent with a process of isolation-by-distance (IBD) in which genetic differentiation

increases with geographic distance as the influence of genetic drift becomes gradually stronger than the homogenizing influence of gene flow. The presence of IBD indicates that *E. invaria* populations have been established in Maryland and Virginia long enough to evolve population structure, and that recent levels of gene flow across the region have not been strong enough to prevent drift and divergence. This supports the hypothesis that *E. invaria* became established in the Mid-Atlantic Piedmont when mayflies moved north into Maryland and Virginia to colonize new stream habitats after the end of the last major regional glaciation (> 10,000 years ago). IBD slopes and intercepts may be used to visualize differences in dispersal abilities among different species or conditions (Peterson & Denno 1998). In this case, Figure 4 shows a uniform rate of change with distance across the sampled populations.

The high gene diversity (heterozygosity) within populations (Table 6) is in keeping with expected levels in outbreeding populations, indicating that significant inbreeding has not occurred even though populations are isolated enough to have evolved moderate to high genetic differentiation. All three measures of diversity (pairwise distance, % polymorphic loci, heterozygosity) were significantly negatively correlated with watershed deforestation. The degree of deforestation in the natal watershed explained more than 50% of the variation in diversity among populations in all three cases, indicating a strong, consistent association of genetic diversity with the total area of forest cover. The strength of the association is due in large part to the fact that most populations in the study fell at one of the two extremes of land use and genetic diversity (forested & more diverse, or deforested & less diverse). This distribution was unavoidable as areas of "intermediate" land use (50-80% forested) are rare in the Mid-

Atlantic Piedmont region, and relatively few streams of this type could be located for the study. However, the few intermediate populations that were found are of particular interest in the analysis of population genetic variation and distribution, because the AFLP data show these intermediate populations are at least as genetically diverse - and by some measures more diverse (Figure 3c) - than the populations in fully forested watersheds. Assignment tests that placed individuals in the "Virginia" or "Maryland" gene pool based on Bayesian analysis of allele frequencies revealed that the intermediate and forested watersheds in Maryland contain the highest proportion of individuals of admixed ancestry (individuals marked with an asterisk (*) in Figure 6) and that populations at the low end of the scale (deforested & less diverse) in Maryland had no strongly admixed individuals (represented by the predominantly green blocks in Figure 6). Admixed individuals are putative migrants carrying alleles from the Virginia gene pool into the Maryland populations. The pattern of admixture shows that while there is a gradient of ancestral mixing across the entire set of sampled populations in Maryland (Figure 6), a relatively small number of individuals (marked as "migrants" with an asterisk in Figure 6) found in watersheds with intermediate-to-high percentage of forested land cover contribute a disproportionately large share of the genetic diversity to these populations.

These results suggest that the higher diversity of the forested streams in Maryland arises from an individual-based mixture of 2 unique regional gene pools, one common and one rare, and that the importance of some migration events should be weighted more heavily than others. The pattern of isolation by distance supports a model of gene flow as a homogenizing influence on the genetic composition of populations is highly relevant to *Inconstans* mayflies. But in a few cases the effects of gene flow are isolated in a small

number of individuals in the receiving population. Evidence from these mayfly populations is that gene flow may leave a distinctive trace from the source population that remains evident over large spatial scales, or large temporal scales, or both.

The specific relationships of admixed and unmixed individuals in spatially discrete populations, the mechanisms by which variation from past migration is retained, and the role of trees as an environmental factor affecting mayfly population diversity are yet to be worked out. Removal of trees has direct and indirect effects on stream and riparian conditions, including loss of adult mayfly habitat and swarming markers, increased water and air temperatures, "flashy" flow regimes, bank erosion, altered stream water chemistry, and changes in localized wind patterns. Although some patches survive and support large populations of mayflies (Chapter 3), reduction in the number and quality of habitat patches may be leading to smaller effective populations and regional loss of genetic diversity. Larger populations support more diverse assemblages of genotypes, including rare alleles found at lower frequencies; and connectivity across small and large spatial and temporal scales enables the transport of alleles among populations in semi-isolated stream and river watersheds. Whatever the specific mechanisms, the implications of these results are that watersheds have been significantly deforested have lost their ability to support and retain historic levels of population genetic diversity in this lineage of mayflies.

Table 1: Sample Sites

County and State:	6-Digit Watershed (HUC#)	8-Digit Watershed (HUC#)	Site ID (see Figure 1)	Population ID and Stream Name
Baltimore Co., Maryland	Gunpowder River (021308)	**Pretty Boy Reservoir (02130806)**	**1. Gunpowder State Park**	1.1 Slip Stream
	Patapsco River (021309)	**Pat R Lower North Branch (02130906)**	**2. Patapsco State Park**	2.1 Daniels Creek
Howard Co., Maryland	Patuxent River (021311)	**Middle Patuxent (02131106)**	**3. Middle Patuxent Env. Area**	3.1 Little Creek 3.2 Right Stream 3.3 T-West Branch
			4. UMD Dairy Farm	4.1 Folly Qtr. Stream 4.2 South Stream
		Rocky Gorge Dam (02131107)	**5. Rocky Gorge Tributary**	5.1 Rocky Gorge Trib.
		Brighton Dam (02131108)	**6. Cattail Creek**	6.1 Hunt Valley Stream 6.2 Miller's Mill Stream
Montgomery Co., Maryland	Middle Potomac River (021402)	**Seneca Creek (02140208)**	**7. Schaeffer Farm**	7.1 Schaeffer Farm 7.2 Black Rock Stream
Appomattox Co., Virginia	James River (020802)	**Appomattox River (02080207)**	**8. Holliday Lake**	8.1 Saunders Creek
Buckingham Co., Virginia		**Slate River (0208020)**	**9. Jamison Creek**	9.1 Big Jamie Creek

Table 2: AFLP adapter and primer sequences

*Eco*RI adapters	5'-AAT TGG TAC GCA GTC-3' 5'-CTC GTA GAC TGC GTA CC-3'
*Pst*I adapters	5'-TGT ACG CAG TCT TAC-3' 5'-CTC GTA GAC TGC GTA CAT GCA-3'
**6-FAM labeled* *Eco*RI primer	(*E1) 5'-GAC TGC GTA CCA ATT CAG-3'
*Pst*I primers	(P1) 5'-GAC TGC GTA CAT GCA GAC A-3' (P2) 5'-GAC TGC GTA CAT GCA GAG A-3'

Table 3: Loci sampled by two primer pairs

Primer pair	Total # fragments	#Polymorphic loci	%Polymorphic loci (PLP)
6-FAM E1/P1	748	671	90%
6-FAM E1/P2	545	424	78%
Total	1293	1095	n/a

Table 4: Watershed characteristics. "Pop ID and Stream Name" refer to Table 1. The columns labeled "deforest", "res+inst", and "ag" are the proportions of deforested, residential+institutional, and agricultural land use within the first-order stream watershed, respectively. "Area" is the first-order stream watershed area in square kilometers.

Pop. ID and Stream Name	***deforest***	***res +inst***	***ag***	***Area (km^2)***
1.1) Slip Stream	0.01	0.01	0.00	1.04
2.1) Daniels Creek	0.39	0.26	0.13	0.93
3.1) Little Creek	0.46	0.38	0.07	0.52
3.3) T-West Branch	0.74	0.71	0.02	1.56
4.1) Folly Quarter Creek	0.83	0.06	0.77	1.04
4.2) South Stream	0.75	0.00	0.75	0.60
5.1) Rocky Gorge Tributary	0.76	0.19	0.57	1.52
6.1) Hunt Valley Stream	0.77	0.25	0.52	2.84
6.2) Miller's Mill Stream	0.79	0.49	0.3	2.07
7.1) Schaeffer's Farm Stream	0.61	0.00	0.61	0.78
7.2) Black Rock Stream	0.52	0.06	0.46	2.08
8.1) Saunders Creek	0.05	0.01	0.04	0.84
9.1) Big Jamie Creek	0.03	0.01	0.02	2.23

Table 5: Matrix of pairwise geographic distance (km, above diagonal) and genetic distance (pairwise Fst, below diagonal). Population ID numbers refer to Table 1.

Pop. ID	1.1	2.1	6.1	6.2	4.2	4.1	3.1	3.2	7.1	7.2
1.1		45.7	60.85	57.69	59.22	58.31	61.22	61.93	96.89	97.13
2.1	0.076		25.21	24.77	15.77	14.12	16.98	17.46	61.17	60.82
6.1	0.096	0.075		4.13	13.99	16.29	15.69	16.84	37.18	36.72
6.2	0.179	0.134	0.166		16.17	18.14	18.27	19.09	39.43	39.38
4.2	0.062	0.025	0.060	0.152		2.34	2.42	3.52	46.1	45.65
4.1	0.072	0.049	0.081	0.158	0.002		2.86	3.57	48.24	47.76
3.1	0.090	0.043	0.042	0.133	0.009	0.014		1.07	45.84	45.42
3.2	0.163	0.100	0.131	0.060	0.093	0.099	0.075		46.1	45.62
7.1	0.109	0.110	0.125	0.203	0.113	0.084	0.105	0.178		0.61
7.2	0.145	0.118	0.174	0.230	0.153	0.165	0.163	0.213	0.120	

Table 6. Estimates of population diversity: Heterozygosity (*hs*), Percent Polymorphic Loci (PLP), and Pairwise Distance (PD) among individuals within populations (PD).

Pop. ID and label	*hs*	PLP (%)	PD
1.1) Slip Stream	0.2561	65.2	126.0
2.2) Daniels Stream	0.2305	67.2	120.5
3.1) Little Creek	0.2293	62.2	116.6
3.2) T-West Branch	0.2377	53.7	110.4
5.3) Folly Quarter Creek	0.2213	61.4	106.3
6.1) Rocky Gorge Tributary	0.2268	61.1	111.1
7.1) Hunt Valley Stream	0.2322	63.7	113.7
7.3) Miller's Mill Stream	0.2180	54.1	102.3
8.1) Schaeffer's Farm Stream	0.2279	59.9	108.3
8.2) Black Rock Stream	0.2489	73.0	137.7
9.1) Saunders Creek	0.2448	82.0	130.6
10.1) Big Jamie Creek	0.2395	74.5	126.3
Average:	**0.2344**	**64.83**	**117.48**

Table 7. Estimates of population structure: θ_B and among-population variation ***Gst***

Groups	θ_B	***95% credible interval***	***Gst***	***%among pop variation***
All sites	0.199	(0.1807, 0.2189)	0.179	89.9
Region 1: Patuxent + Patapsco (MD)	0.082	(0.0737, 0.0916)	0.070	85.4
Region 2: Potomac (MD)	0.059	(0.0384, 0.0801)	0.031	52.5
Region 3: MD excluding Gunpowder	0.097	(0.0872, 0.1062)	0.086	88.7
Region 4: MD including Gunpowder	0.179	(0.1671, 0.1912)	0.163	91.1
Region 5: James + Appomattox (VA)	0.030	(0.0166, 0.0466)	0.016	53.3

Figure Captions

Figure 1: Samples Sites in (a) Maryland and (b) Virginia. Site numbers refer to Table 1.

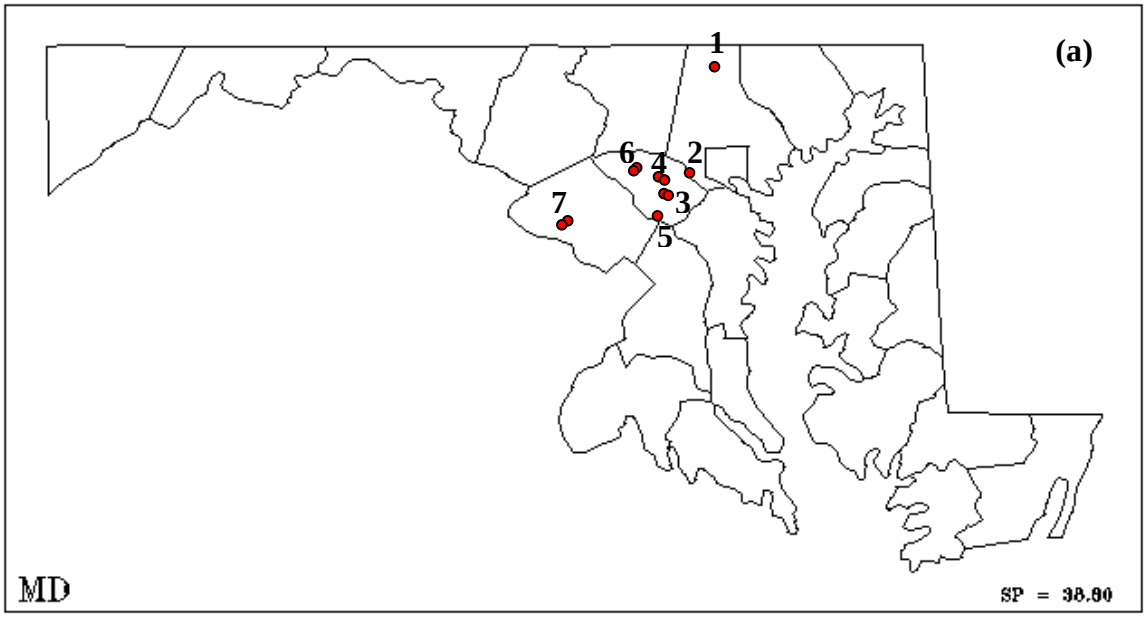
Figure 2. Correlation of pairwise distance estimates from 2 primer pairs.

Figure 3: Linear regressions of population diversity on % watershed deforestation.
a) Heterozygosity (*hs*) Hickory, f-free model (Holsinger *et al.* 2002)
b) %Polymorphic loci (PLP) AFLP-SURV (Vekemans 2002)
c) Pairwise distance (PD) Arlequin (Excoffier *et al.* 2005)

Figure 4: RMA regression of genetic distance to log-transformed geographic distance for 10 Maryland populations.

Figure 5: Individual assignment to inferred populations.

Figure 1: Samples Sites in (a) Maryland and (b) Virginia. Site numbers refer to Table 1.



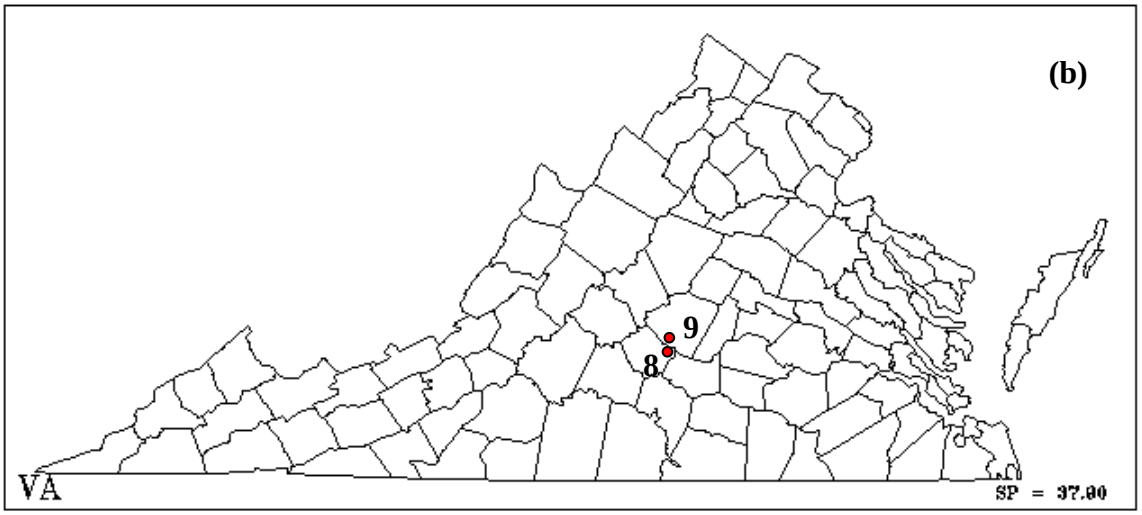


Figure 2. Correlation of pairwise distance estimates from 2 primer pairs

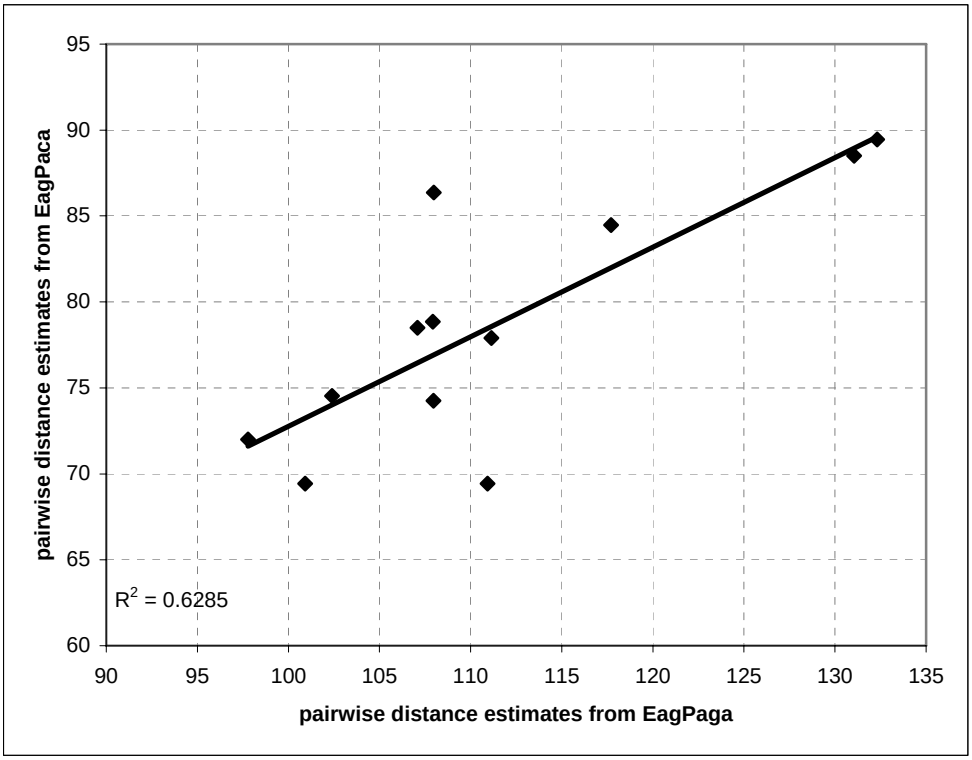
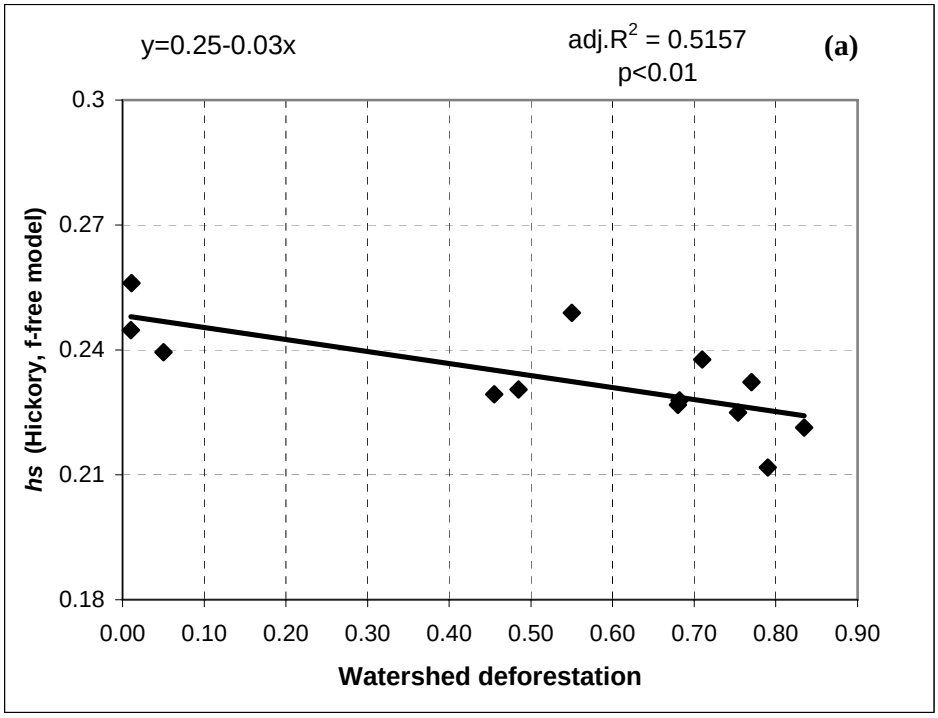


Figure 3: Linear regressions of population diversity on % watershed deforestation.

a) Heterozygosity (*hs*) Hickory, f-free model (Holsinger *et al.* 2002)

b) %Polymorphic loci (PLP) AFLP-SURV (Vekemans 2002)



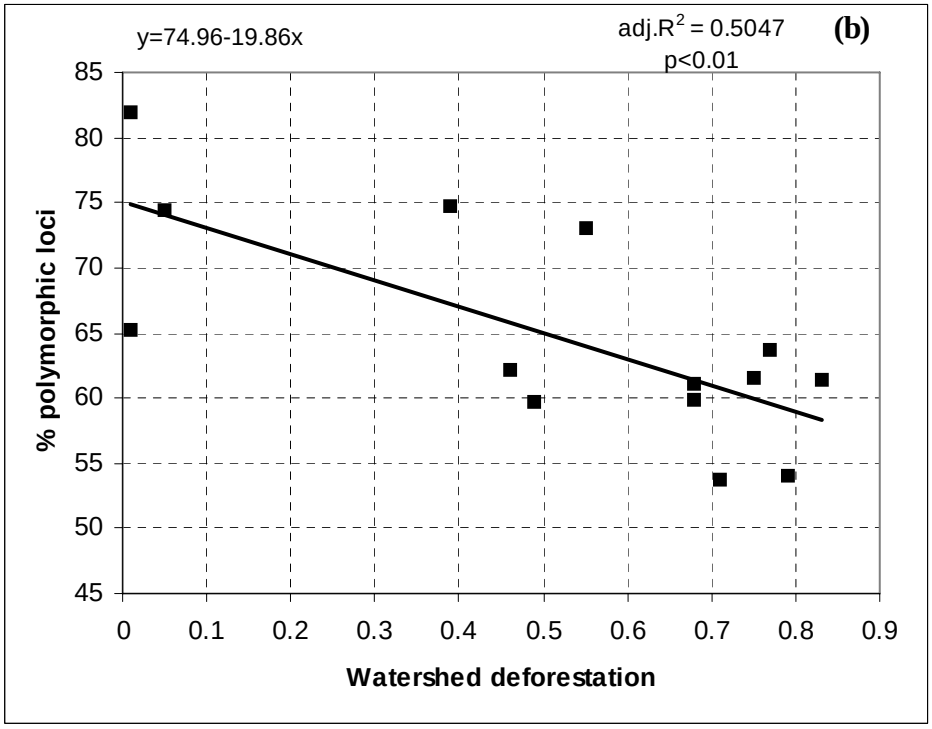


Figure 3: Linear regressions of population diversity on % watershed deforestation **(continued).**

c) Pairwise distance (PD) Arlequin (Excoffier *et al.* 2005)

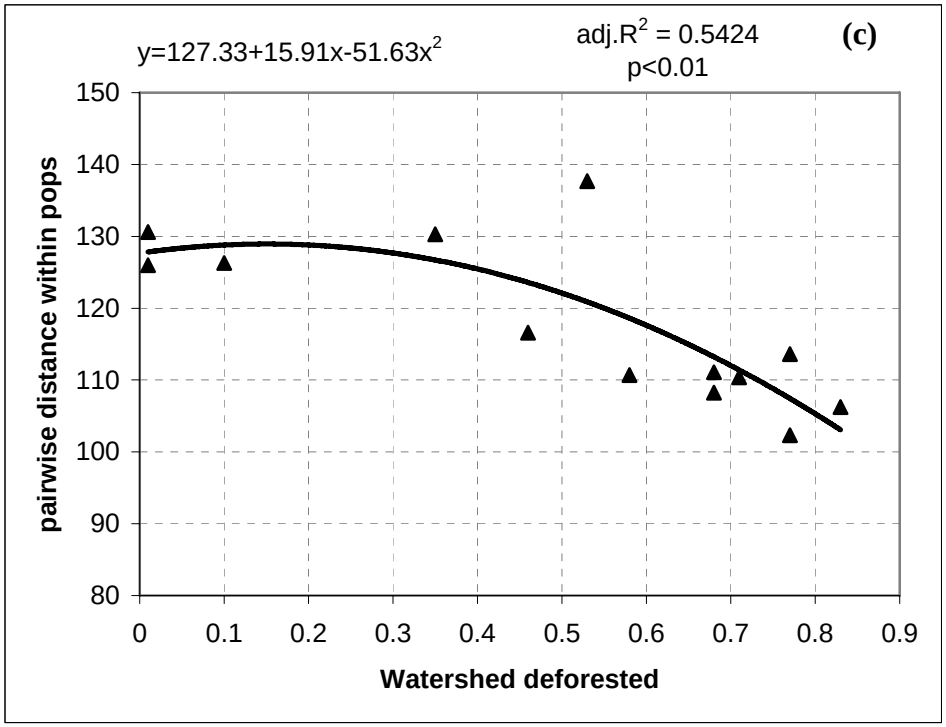


Figure 4: RMA regression of log-transformed gene flow ($\hat{M}$ = ¼ (1/Fst – 1)) over log-transformed geographic distance (km). The negative linear relationship indicates a significant association of genetic distance with geographic distance. Genetic distance is plotted here as $\hat{M}$, the estimated level of gene flow in an island model at equilibrium (Slatkin 1993).

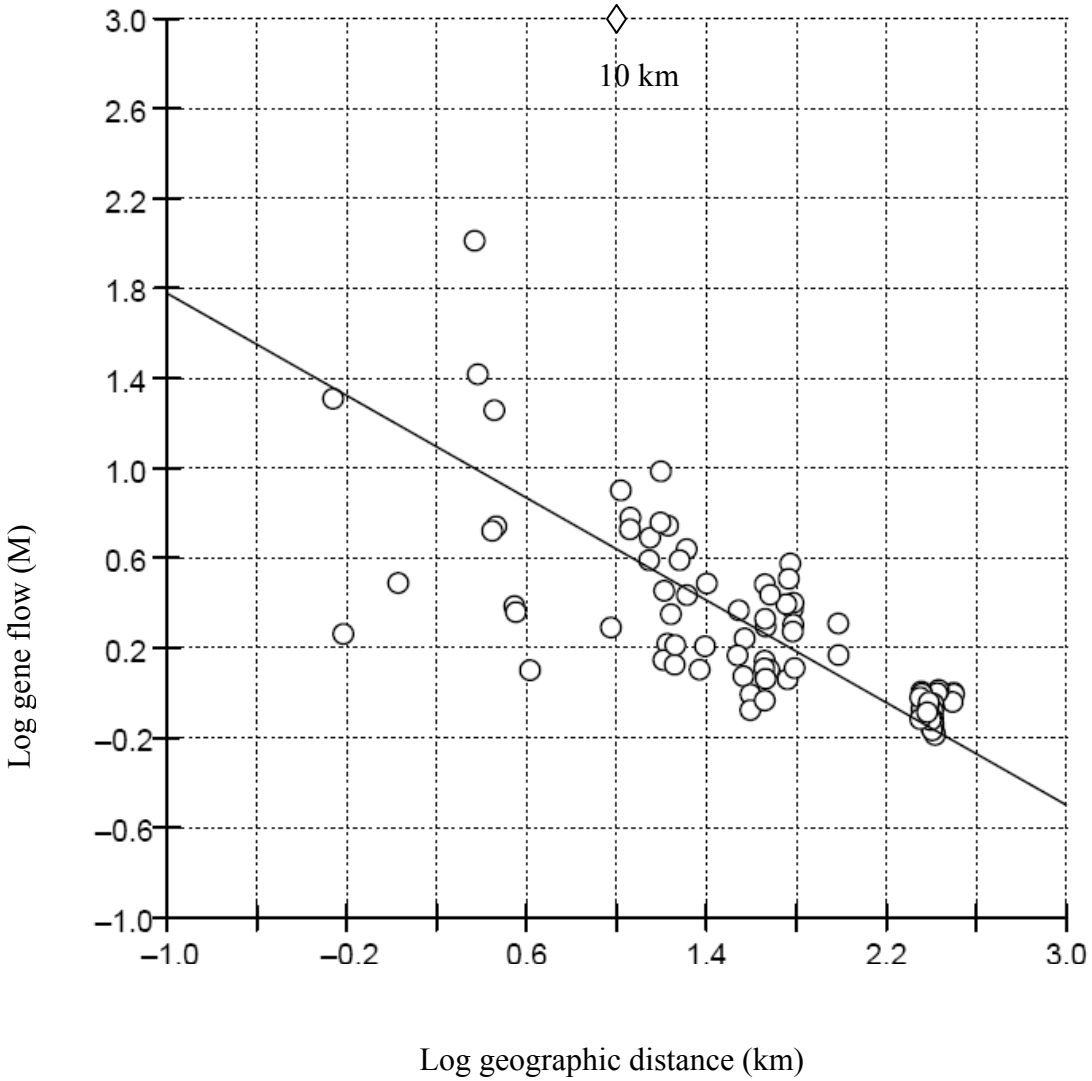
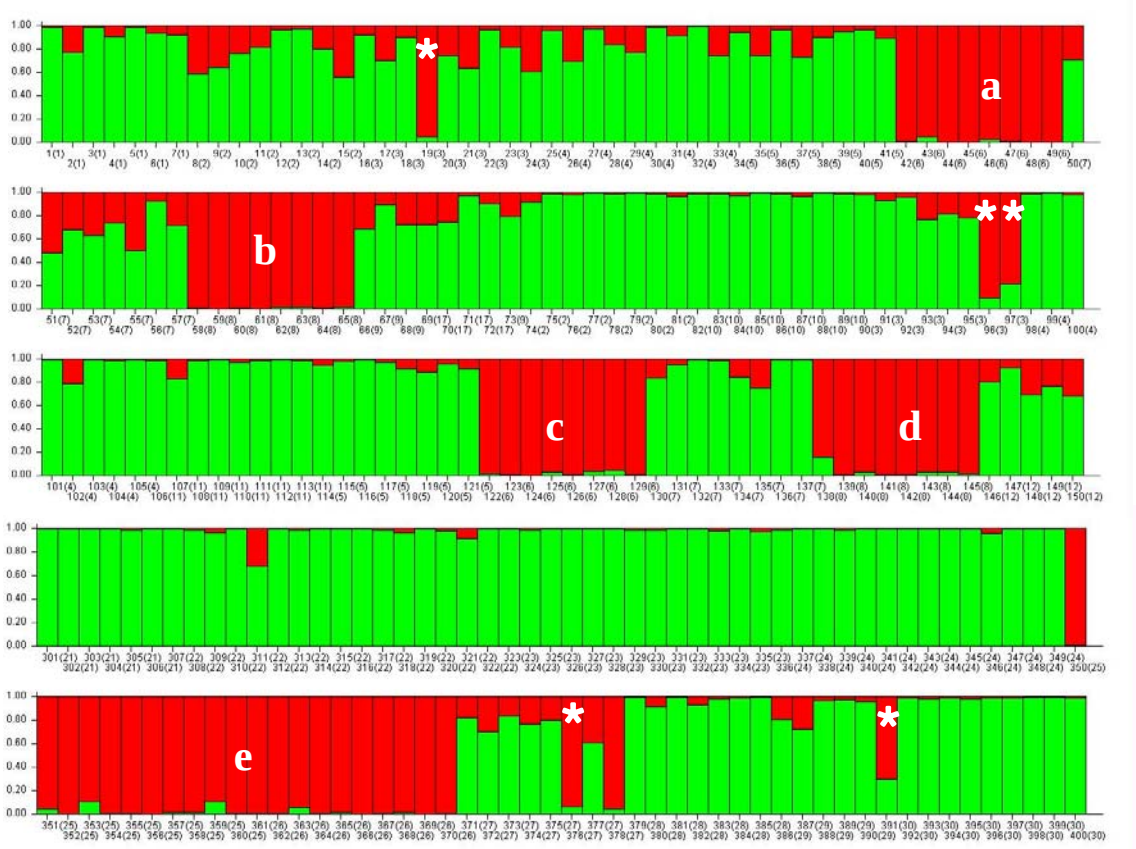


Figure 5: Individual assignment to inferred populations. Red areas represent Virginia ancestry; green represents Maryland ancestry. Samples sites in Virginia are the red blocks labeled a-e; all other individuals are from samples sites in Maryland.

(*hypothesized migrants disproportionately influencing population diversity)



CHAPTER III: Mayfly Population Density, Diversity and Persistence Through Drought In Disturbed Headwater Streams

Abstract

I assessed the effects of drought and deforestation on the population density, diversity, and persistence of a mayfly (*Ephemerella invaria* Walker, 1853) in 24 first-order streams across 9 headwater stream networks in Maryland and Virginia over 4 successive years (2001-2004). I present differences in density and local extinction in forested versus deforested headwater streams, and report estimates of population genetic diversity from amplified fragment length polymorphism (AFLP) before and after a severe drought that caused some of the sampled streams to go dry. I predicted that mayfly density would be higher and population extinction rates lower at forested sites compared with deforested (agricultural and residential) sites, but found no difference in initial density at forested and deforested sites (2001-2002) and no difference in the level of population decline across all sites by the end of the drought (spring 2003). However, one year after the drought had ended (spring 2004), population density was significantly higher in forested streams compared with streams flowing through agricultural and residential areas. Further, while only 1 of 11 populations at forested sites remained extirpated in 2004, populations in 4 of the 13 deforested streams were extirpated at the end of the study. These results suggest that recovery and re-colonization following a major regional disturbance was more successful in the forested stream networks than in the deforested networks. To examine the population genetic effects of the demographic decline and post-drought recovery, I measured genetic diversity in nine surviving and recovered populations using amplified fragment length polymorphism (AFLP) markers

and found little change relative to the estimates of pre-drought average panmictic diversity reported in chapter 2. But individuals previously identified as migrants or hybrids are more rare, and may represent the most important loss of diversity due to demographic and environmental factors during the period. Post-drought differences in the population genetic composition at a re-populated headwater stream that had experienced dry-down and extirpation suggests the primary source of colonists may have been the river mainstem, as previously significant population structure between the mainstem and headwaters was reduced after the drought. I discuss the implications of the combined effects of bottleneck, habitat loss, and environmental disturbance on the genetic composition and persistence of these populations.

Introduction

Analysis of the population genetic structure of the mayfly *Ephemerella invaria* in chapter 2 found that the current pattern of population structure is generally consistent with a process of isolation-by-distance (IBD) in which genetic differentiation increases with geographic distance. Moderate to high levels of heterozygosity were found at all sites and three measures of within-population genetic diversity (percentage of polymorphic loci, pairwise distance among individuals, and heterozygosity) were inversely related to the degree of watershed deforestation.

During the sampling period, the study populations were exposed to a severe regional drought that caused some headwater streams to dry down. In this chapter, I consider the combined effects of anthropogenic disturbance (deforestation) and environmental disturbance (drought) on the population density, diversity, and persistence

of *E. invaria* in the study region of central Maryland and Virginia.

Hydrologic drought is broadly defined by Yevjevich (1977) as a "period of below average water content in streams, reservoirs, ground-water aquifers, lakes and soils." Direct effects (e.g., loss of water, flow, habitat, and dispersal routes) and indirect effects (changes in interspecific interactions and food resources) of hydrologic drought can reduce population densities and species richness, alter community composition, trophic structure, or life cycle schedules (Lake 2003, Boulton 2003) but actual perturbation of stream ecosystems by drought varies widely across regions and time periods (Humphries & Baldwin 2003, McMahon & Finlayson 2003). The frequency and duration of the reduction in surface and subsurface water supply are the key variables in the definition of a hydrologic drought (Wilhite 2000) and its effect on stream biota (Humphries & Baldwin 2003). Flow disturbances are characteristic of lotic ecosystems and many aquatic organisms have adapted to drought by developing physiological resistance to desiccation in one or more life stages, or through behavioral use of habitat refugia (Magoulick & Kobza 2003, Williams 1996, Clinton *et al.* 1996, Del Rosario & Resh 2000). In extreme drought, even populations of species adapted to variable flow conditions may be eliminated or greatly reduced (e.g., Resh 1992). Recovery of stream communities in re-wetted streams following supra-seasonal drought is sometimes surprisingly rapid (e.g., Churchel & Batzer 2006, Caruso 2002) but succession and lags in recovery of previously abundant species has also been observed (e.g., Smock *et al.*1994, Boulton & Lake 1992) and little information is available on the long-term population effects of drought on stream invertebrates.

Here I measure population density, local extinction, and stream recolonization in the two types of watershed following a severe drought that reduced flow in all streams and caused some streams to dry completely down. I also measure the effect of the environmental disturbance on the population genetic diversity at 9 stream sites, relative to pre-drought diversity estimates reported in chapter 2. My expectations were that population density would higher, extinction rates lower, and recovery faster in more heavily forested streams compared with those at deforested (agricultural and residential) sites that were already stressed by local disturbance and thus more susceptible to the disturbance effects of the drought.

Methods and Materials

Drought Conditions

The Maryland State Climatology Office website *(http://www.atmos.umd.edu/~climate)* states that the period from September 2001 through August of 2002 was Maryland's second driest 12 months in the 108-year record of precipitation history in Maryland, exceeded only by the "dustbowl" drought of 1930-1931. The US Drought Monitor website, a national partnership between the Drought Mitigation Center at the University of Nebraska, government agencies, and state climatologists *(http://drought.unl.edu/dm)*, places the beginning of Maryland's drought in May 2001 and the termination in December 2002, with the period of spring and summer 2002 classified as "exceptional drought". A summary of the monthly values of Palmer Drought Severity Index (PSDI), a long-term index of drought period and severity that (Palmer 1965, Byun and Wilhite 1999), for the sampling period (Figure 7) shows that a continuous period of severe-to-

extreme drought persisted from February through August 2002**,** followed by a period extremely wet conditions through 2003 and unusually wet conditions through 2004.

The 2001-2002 drought in Maryland and Virginia caused physical disturbances at the study sites that ranged from reduced flow to complete dry-down in the headwaters. The drought effects reported here are from temperate, perennial first-order streams with watershed areas ranging from 0.5 to 3.0 km^2. I measured differences in mayfly (*Ephemerella invaria,* Ephemerellidae) population density and genetic diversity in forested and deforested watersheds before and after a severe drought, and compared the rate of recovery in the two types of watershed.

Study Organism

The naturally patchy distribution of headwater stream habitat is reflected in the population distribution of *E. invaria* in the states of Maryland and Virginia, USA. A previous phylogenetic study of closely-related species in the genus *Ephemerella* (Chapter 1) determined that the *E. invaria* populations in headwater streams in this region comprise a single genetic lineage, hereafter referred to as "*Inconstans*".

Study sites

A total of 24 headwater streams in 9 headwater stream networks were sampled. The process used to select sites for the study is described in chapter 2. Ten streams were sampled in 2001, 20 streams in 2002, and 24 streams in both 2003 and 2004. The names and locations of the study sites, which fall within 5 major river watersheds in Maryland and Virginia, are provided in Table 1. Each stream network consists of 1-4 adjacent

headwater streams containing one or more populations of *E. invaria*. All study sites are located within the region of Central Piedmont between 37°20' and 39°20' latitude, bounded to the west by the Appalachian mountains and to the east by the Coastal Plain. Although lengths and flow regimes vary among the streams due to differences in local topography, groundwater sources, and land use, a typical stream in this study drains an area < 1 km^2 with baseflow discharge < 0.03 m^3/s.

Sampling

In 2001, 2002, and 2004, nymph samples were collected using moss-packs (colonizing samplers) consisting of a fixed amount of dried moss enclosed in plastic mesh bags and tied with string to roots or stakes along the stream margin for a period of 3 weeks in March and April, when late instar *E. invaria* nymphs are present in the stream margins. Moss-packs are designed to move freely with stream flow to imitate natural moss or root-wad habitats. They are readily colonized by *E. invaria* and other aquatic invertebrate taxa. Eight moss-packs were placed in each stream, positioned in pairs along a 75 m reach so that a total of 4 sub-samples were taken in each stream.

Samples were bagged in stream water and sorted while specimens were still alive. All ephemerellid mayflies were identified to species using Allen & Edmunds' key (1965), counted, and stored in 100% ethyl alcohol at –20°C. When a stream sample contained fewer than 16 individuals of *E. invaria*, that stream was re-sampled with a D-frame net to increase the size of the sample available for population genetic analysis. The extra samples were labeled, stored separately from the moss-pack samples, and excluded from the population density counts.

In 2003 nymph samples were collected with a D-frame net. From a comparison of samples taken using both methods in one stream, active search with a D-frame net produced larger sample counts and thus would overestimate the density relative to the mosspack samples. To make the D-frame samples comparable to the moss-pack samples for categorical estimates of population density, D-frame sampling for 2003 season was constrained to 3 twenty-five meter sections selected at random from a 150 meter stream reach. All suitable habitats in the substrate and stream margins within the 3 randomly selected sections were sampled extensively. Processing of samples in 2003 was done as in 2001, 2002, and 2004, described above.

Population density and persistence

Nymph sample counts were converted to a categorical variable with 4 levels: none (sample count=0), rare ($0 <$ sample count < 10), common ($10 \leq$ sample count < 20), and abundant (sample count ≥ 20). The counts in each density category were plotted to visually check for trends in the density distribution in forested versus deforested streams within each year. The density categories were then combined to create two broader density categories: low (sample count < 10) and high (sample count ≥ 10), to compare the densities in forested and deforested streams using Fisher's Exact Test. A separate statistical test was conducted for each of the last 3 years (2002, 2003, 2004). The results of 2002 reflect density prior to the peak drought; the results of 2003 reflect the population response to the drought peak (summer 2002); and the results of 2004 reflect the population recovery one year after the end of the drought.

Genetic analysis

A portion of the mitochondrial DNA (mtDNA) cytochrome oxidase (CO) I gene was sequenced, using the methods described in chapter 1, in 16-24 individuals from 15 populations across the range of sample sites to verify that the sampled populations were from the genetic lineage *Inconstans*. The AFLP primers and methods described in chapter 2 were then used to generate a data matrix of individuals from a random sample of individuals in the populations noted with an asterisk (*) in Table 1.

Results

Population density and persistence

The population density distribution for each year in (a) all streams; (b) forested streams only; and (c) deforested streams only, is described and plotted in Figure 1. The plot of combined sites (Figure 1a) shows the general trend of population decrease during the drought, followed by population increase during recovery. The apparent symmetry of the combined response is a composition of inverse patterns of response by forested streams and deforested stream populations (Figures 1b and 1c). One year after the drought (2004), 90% of forested streams were classified as having high (= abundant + common) mayfly density, none were classified rare, and the population in 1 of the 10 streams (10%) was extinct. In the deforested streams, 50% of streams were classified as high mayfly density, 21% were rare, and populations in 4 of 14 streams (29%) were extinct. Fisher's Exact Tests of categorical density in forested and deforested sites within each year (Table 3) show that the population density does not differ between forested and

deforested sites in years 2002 or 2003 (p>0.4 and p>0.1), indicating that streams could not be distinguished by land use (forested or deforested) at the start of the study or at end of the drought. However, one year after the drought had ended (spring 2004) the proportion of streams with high population density was significantly greater in forested than in deforested streams (p<0.05).

Population genetic diversity

Estimates of post-drought population heterozygosity (*hs*, Table 3) were generated in Hickory (Holsinger 2002) as explained in chapter 2. Plots of pre- and post-drought heterozygosity regressed on watershed deforestation are shown in Figure 2a. The post-drought samples are more variable (y=0.25-0.03x, adj. R^2 =0.29, p<0.1) but the regression line and significance are unchanged from the pre-drought samples (y=0.25-0.03x, R^2 =0.53, p<0.01). Treating the pre- and post-drought samples as sub-samples of the same statistical (and genetic) population, average heterozygosity across time produces a better estimate of the true population heterozygosity at each site (Table 3 and Figure 2b). In Figure 2b, the regression line is unchanged but the proportion of variance explained by the independent variable (% deforestation) was increased (adj. R^2 =0.76, p<0.001). Figure 3a plots pre- and post-drought estimates of pairwise distance among individuals within sites as an alternative measure of genetic diversity. The sign change of the regression coefficients suggests that for this measure of genetic diversity, stream sites with intermediate levels of deforestation (30-60% deforested) may have been more seriously affected by drought than either the most heavily forested or deforested stream sites. But when analyzed as sub-samples of the same statistical and genetic population

(Figure 3b), the trend in average pairwise distance across time is similar to the trend in the relationship of heterozygosity to deforestation in these small watersheds (adj. R^2 =0.86, p<0.001).

Isolation by distance

Seven surviving populations in Maryland exhibit a weak (R^2=0.34, p<0.03) correlation of genetic and geographic distance (Table 4 and Figure 4) after the drought.

Assignment Tests

Structure identified 2 source populations, one in Maryland and the other in Virginia, after the drought. The proportion of Virginia ancestry assigned to the Maryland populations (Figure 5) was reduced from 0.0659±0.0262 (range 0.006-0.286) before the drought to 0.0165±0.0042 (range 0.003-0.048) after the drought (Figure 5). The number of admixed individuals in Maryland with >50% Virginia ancestry was reduced from 14 in the pre-drought samples (chapter 2) to 1 individual in the post-drought samples.

Population structure of a recolonized stream.

Samples before and after drought of one creek that had dried down completely revealed a shift in its genetic composition. Figure 6 represents the pairwise genetic distances among samples from three co-located populations: two populations from adjacent headwaters (one that dried down and one that stayed wet throughout the drought) and one population from the mainstem upstream of the headwater confluences. The green bars are the genetic distance between the two adjacent headwater streams,

before and after the drought. The orange and white bars are the pairwise genetic distances between the mainstem and each of the two headwaters (orange="dry" stream, white="wet" stream). The strong genetic differentiation (population structure) present before the drought was reduced to 0 for the "dry" (recolonized) stream, but unchanged for the "wet" stream, with respect to the mainstem, suggesting that the mainstem was the source of new colonists of the dry stream. Divergence from the adjacent headwater, another potential source for recolonization of the dry headwater, also was also reduced.

Discussion

Population density and persistence

The results of this study show that population recovery and habitat re-colonization following a major regional disturbance was rapid overall but more successful in forested watersheds than in deforested watersheds. The difference in recolonization rates could be because dispersal from surviving populations to uninhabited patches was more effective in forested stream networks, resulting in a higher probability of re-colonization as well as a larger founding population in these streams. Or, it could be that refugia in the forested sites (e.g., in the hyporheic zone) provided protection to a small number of individuals who were able to regenerate large population sizes in a single generation. These findings are consistent with studies that have found habitat type to be a significant predictor of local extinction, even after the effect of regional distribution has been removed (e.g., Korkeamaeki & Suhonen 2002).

Major patterns and trends in the distribution of genetic diversity among the study populations were not changed by the drought (Figures 2 and 3, Table 3). Isolation by

distance and the correlation of diversity with loss of tree cover were similar to the pre-drought conditions. One measure of genetic diversity, pairwise distance, showed loss of diversity in populations that previously had a high number of admixed individuals (individuals identified as migrants in chapter 2). This may be seen in Figure 3a, where the endpoints of the trend line (forested/more diverse, deforested/less diverse) are stable but intermediate locations are highly changeable; and in Figure 5, where the proportion of migrants (i.e. individuals in Maryland but with >50% Virginian ancestry) are plotted before and after the drought. Although census population sizes recovered rapidly after the drought, sites suffering the greatest loss of migrants did not recover to pre-drought levels of diversity. The lower level of admixture in the Maryland populations after the drought (Figure 5) is most likely a direct result of the reduction in demographic size during the drought. As these individuals contributed a disproportionately large share of genetic diversity, their loss may be the most serious population genetic consequence of the drought.

Reach-scale migration

I had the opportunity to observe the dry-down, re-wetting, and recolonization of one headwater stream, Little Creek, in the Middle Patuxent Environmental Center, in Howard Co., MD. Samples taken before the drought in Little Creek and an adjacent headwater that did not go dry during the drought (Right Stream), were compared with each other and with samples taken from the mainstem (Middle Patuxent River) upstream of the confluences of the two headwaters after the drought. The estimates of pairwise Fst (estimates of population structure, or differentiation between pairs of populations) are

show in Figure 6. The level of population structure between the continuously wetted stream and the mainstem (white bar) was not affected by the drought. However, significant population structure present between the dry stream (Little Creek) and the mainstem before the drought (orange bar) has been eliminated, suggesting that the insects re-colonizing Little Creek migrated into the headwaters from the mainstem. There is also a reduction in the population structure between the two headwaters (Little Creek and the Right Stream, green bar), indicating that some local migration may have occurred from the adjacent headwater as well.

My analysis of population structure and diversity shows that populations of *Ephemerella invaria* do not form a single panmictic population but have diverged significantly in a stepping-stone or isolation by distance pattern across the central Piedmont regions of Maryland and Virginia. However, gene flow is clearly occurring at both large and small scales. Migration and gene flow have the expected homogenizing effect in populations with lower levels of diversity (deforested sites), but may actually be increasing the degree of differentiation among populations with higher diversity (forested sites). This counter-intuitive result could be explained by a metapopulation model in which populations diverge, go extinct, and then are re-established by migrants from other patches. At the larger scale, the population dynamics appear to fit a source-sink metapopulation model, with the source population in Virginia and the sink populations in Maryland and a small but important amount of unidirectional gene flow between them. Future studies should focus on the use of metapopulation models to better understand the interaction of deforestation and environmental disturbance as they relate to the extinction and migration in mayfly population in headwater streams.

Table 1: Sample Sites and Years Sampled

County and State (USA):	**8-Digit Watershed (HUC#)**	**Headwater stream network :**	**Number of streams sampled:**			
			2001	2002	2003	2004
Baltimore Co., MD	Patapsco River - Lower North Branch (02130906)	Daniels Creek*	-	3	3	3
Howard Co., MD	Middle Patuxent River (02131106)	MPEA*	3	3	3	3
		Homewood	-	1	2	2
		UMD CMREC*	1	3	3	3
	Rocky Gorge Dam (02131107)	Rocky Gorge*	1	1	1	1
	Brighton Dam (02131108)	Cattail Creek*	2	3	3	3
Montgomery Co., MD	Seneca Creek (02140208)	Little Seneca *	1	3	3	3
Appomattox Co., VA	Appomattox River (02080207)	Saunders Creek*	2	3	3	3
Buckingham Co., VA	Slate River (0208020)	Jamison Creek*	-	-	3	3
		Total per year:	10	20	24	24

* Sampled for genetic diversity

Table 2: Fisher's Exact Test of density across sites, within years

Site (Forested, Deforested) by Density (Low=None + Rare, High=Common +Abundant)

a. 2002	Low	High
Forested	2	6
Deforested	3	9

a) 2002: No difference in density by site during drought ($p>0.4$)

b. 2003	Low	High
Forested	4	6
Deforested	10	4

b) 2003: No difference in density by site at the end of the drought ($p>0.1$)

c. 2004	Low	High
Forested	1	9
Deforested	7	7

c) 2004: Significant difference in density one year after drought ($p<0.05$)

Table 3: Pre- and Post-drought Heterozygosity (*hs*). Post-drought cells marked with "×" indicate extirpation of the population during the drought.

Major River Watershed, Headwater Label	*Post-drought hs*	*Pre-drought hs*	*Average hs across years*
Gunpowder River, Slip Stream	0.2561	×	0.2562
Patapsco River, Daniels Stream	0.2305	0.2392	0.2392
Middle Patuxent River, Little Creek	0.2293	0.2316	0.2305
Middle Patuxent River, T-West Branch	0.2377	×	0.2377
Middle Patuxent River, Folly Quarter Stream	0.2213	×	0.2213
Middle Patuxent River, South Stream	0.2250	0.2231	0.2240
Patuxent River, Rocky Gorge Tributary	0.2268	0.2245	0.2256
Patuxent River, Hunt Valley Stream	0.2322	0.2080	0.2201
Patuxent River, Miller's Mill Stream	0.2118	0.2371	0.2245
Potomac River, Schaeffer's Farm Stream	0.2279	×	0.2279
Potomac River, Black Rock Stream	0.2489	0.2452	0.2470
Appomattox River, Holliday Lake	0.2448	0.2500	0.2474
James River, Big Jamie Creek	0.2395	0.2322	0.2358

Table 4: Matrix of geographic distance (km, above diagonal) and genetic distance (Fst, below diagonal)

Pop. ID	6.2	4.2	3.1	7.2	2.1	6.1	3.2
6.2		16.17	18.27	39.38	24.77	4.13	19.09
4.2	0.040		2.42	45.65	15.77	13.99	3.52
3.1	0.040	0.002		45.42	16.98	15.69	1.07
7.2	0.210	0.256	0.251		60.82	36.72	45.62
2.1	0.170	0.179	0.204	0.118		25.21	17.46
6.1	0.105	0.125	0.131	0.278	0.234		16.84
3.2	0.102	0.095	0.086	0.283	0.221	0.049	

Figure Captions

Figure 1: Density Distribution by Year
Counts represent the density distribution for each year in:
(a) all streams, (b) forested streams, and (c) deforested streams.

Figure 2: Linear regressions of heterozygosity on percentage watershed deforestation.
a) Pre- and Post-drought Heterozygosity within sites (*hs*)
b) Average Heterozygosity within sites across all years (A*hs*)

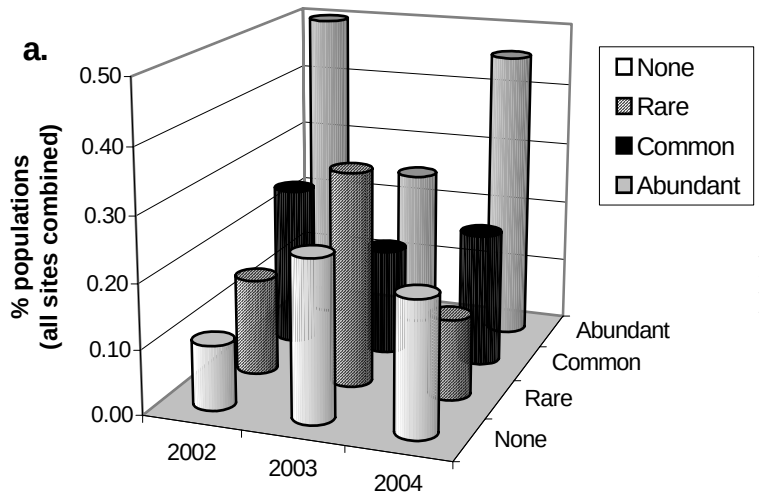
Figure 3: Linear regressions of pairwise distance among individuals within sites on percentage watershed deforestation.
a) Pre- and Post-drought pairwise distance within sites (PD)
b) Average pairwise distance within sites across all years (APD)

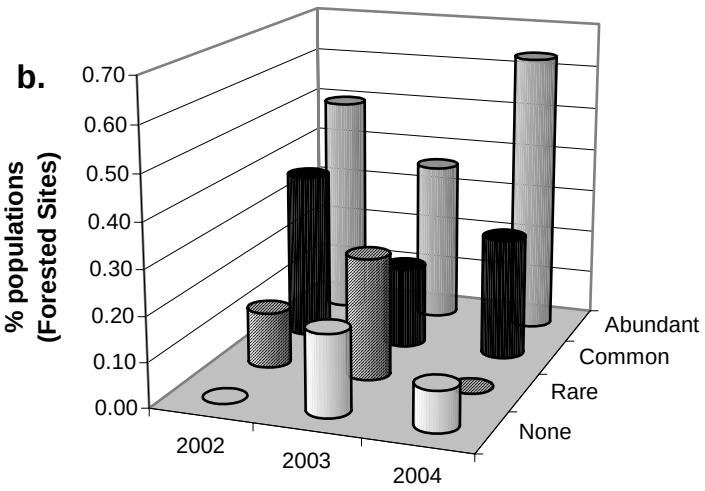
Figure 4: RMA regression of log-transformed gene flow ($\hat{M}$ = ¼ (1/Fst – 1)) over log-transformed geographic distance (km). The negative linear relationship indicates a significant association of genetic distance (here plotted as $\hat{M}$, the estimated level of gene flow in an island model at equilibrium (Slatkin 1993)) with geographic distance.

Figure 5: Structure of (a) pre-drought and (b) post-drought inferred populations. Each vertical line represents a sample site. The percentage of membership in the "Maryland" genetic population is in blue; the percentage of membership in "Virginia" population is in red; the state in which the site is located is on the x-axis. Maryland populations with a large number of migrants have a higher membership in the "Virginia" group (red).

Figure 6. Changes in population structure at a recolonized site following the drought.
Pre-drought samples of the "Dry Stream" were taken from the headwaters prior to dry-down and are compared with the mainstem (orange) and adjacent headwater (green) before the drought. The adjacent headwater to the mainstem pairwise Fst is shown in white.
Post-drought samples of the "Dry Stream" were taken from the recolonized headwaters and are compared with the mainstem (orange) and adjacent headwater (green) after the drought. The adjacent headwater to the mainstem pairwise Fst is shown in white. The negative genetic distance (orange) is interpreted as zero population structure.

Figure 7: Summary of Palmer Drought Index (PDI) values in Maryland 2001-2004





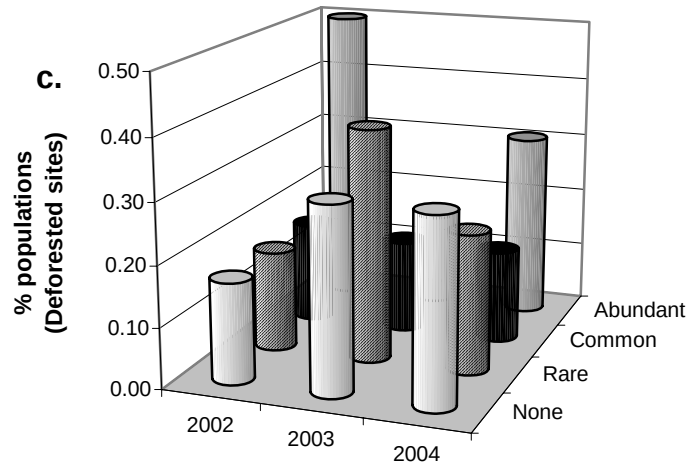


Figure 1:
Density Distribution by Year

Counts represent the density distribution for each year in: (a) all streams, (b) forested streams, and (c) deforested streams.

a. All sites

	2002	2003	2004
None	2	6	5
Rare	3	8	3
Common	5	4	5
Abundant	10	6	11
Total:	20	24	24

b. Forested Sites

	2002	2003	2004
None	0	1	1
Rare	2	3	0
Common	3	3	3
Abundant	3	3	6
Total:	8	10	10

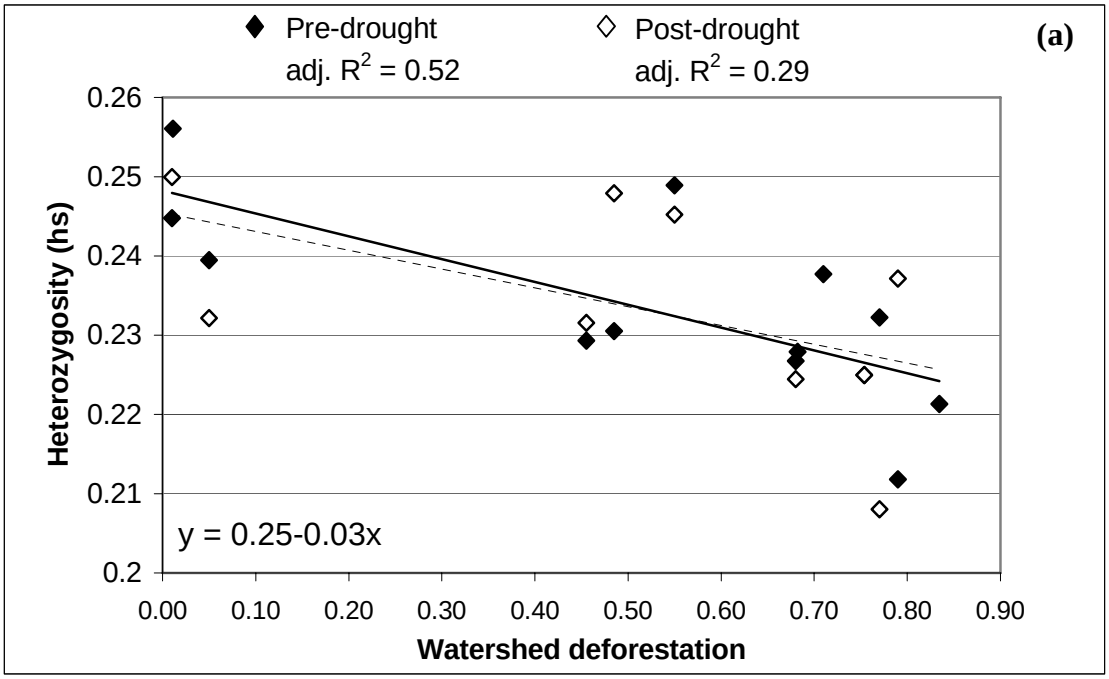
c. Deforested Sites

	2002	2003	2004
None	2	5	4
Rare	1	5	3
Common	2	1	2
Abundant	7	5	4
Total:	12	14	14

Figure 2: Linear regressions of heterozygosity on % watershed deforestation.

a) Pre- and Post-drought Heterozygosity within sites (*hs*)

b) Average Heterozygosity within sites across all years (A*hs*)



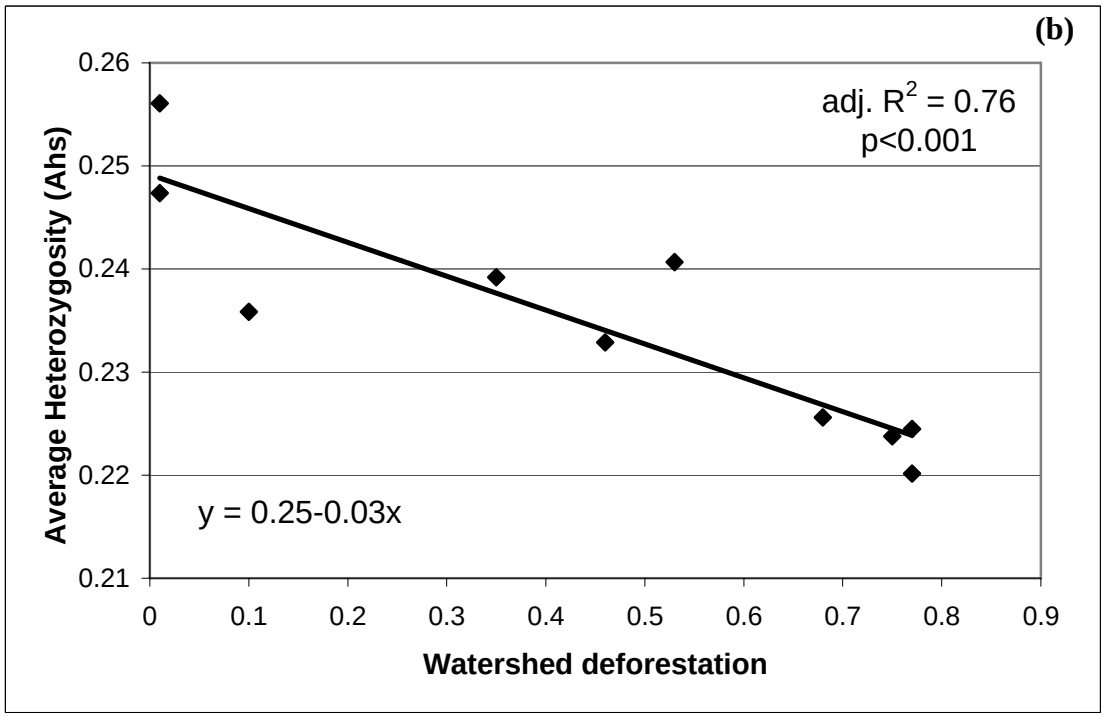
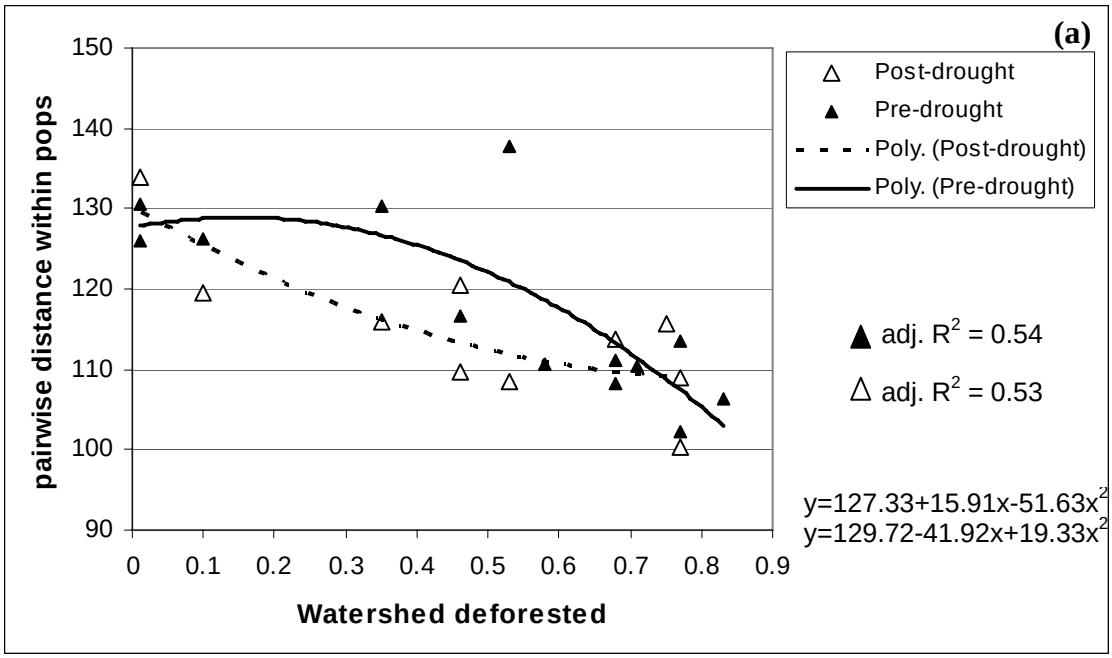
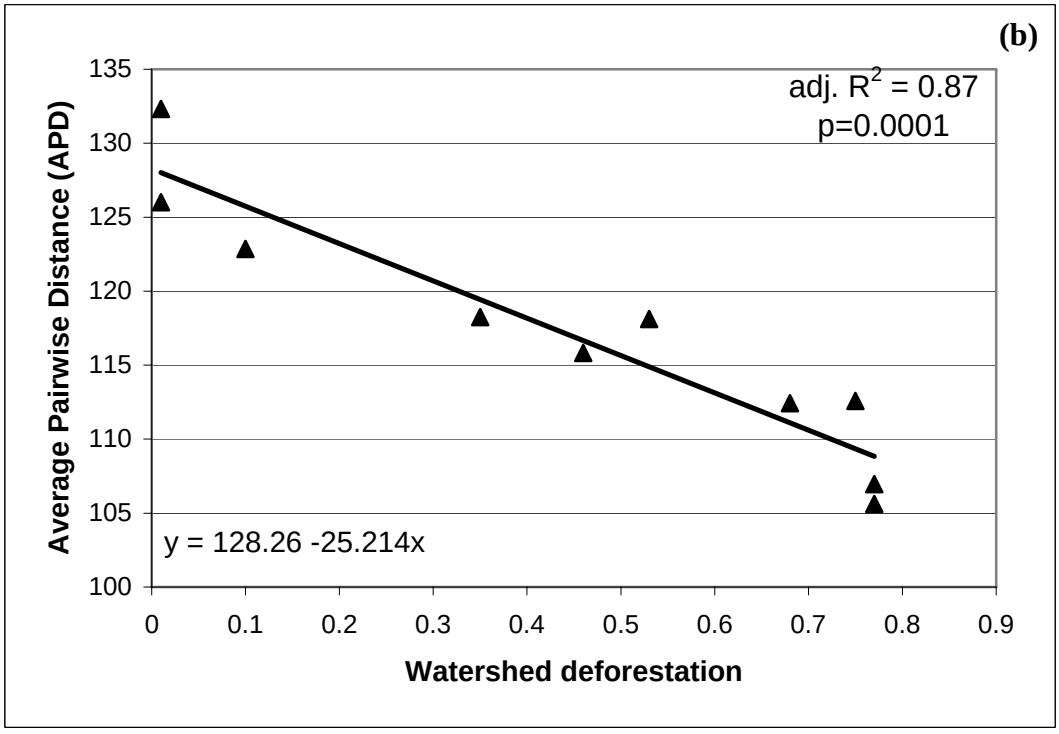


Figure 3: Linear regressions of pairwise distance among individuals within populations (PD) on percentage watershed deforestation.

c) Pre- and Post-drought Pairwise Distance within sites (PD)

d) Average Pairwise Distance within sites across all years (APD)





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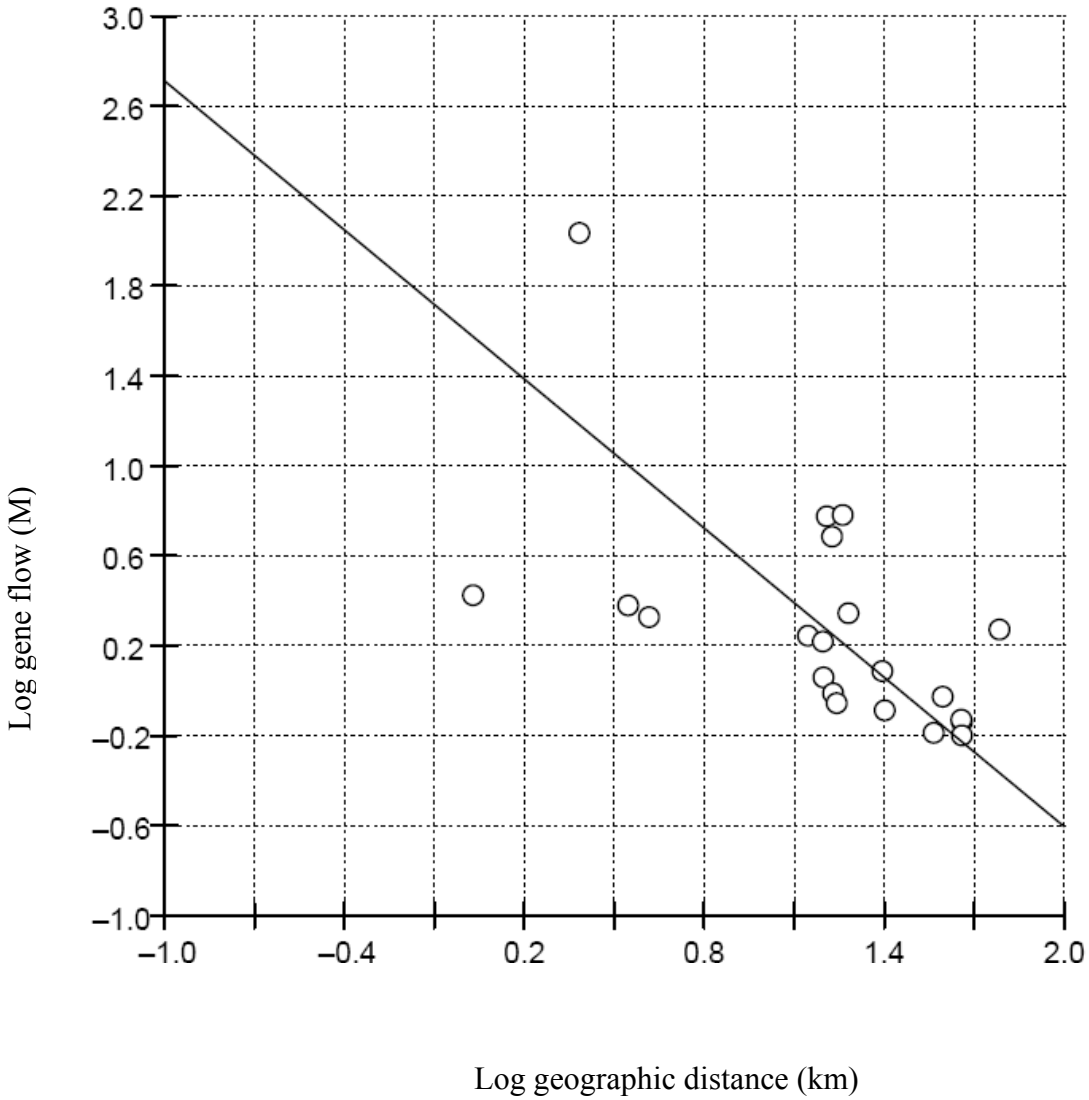

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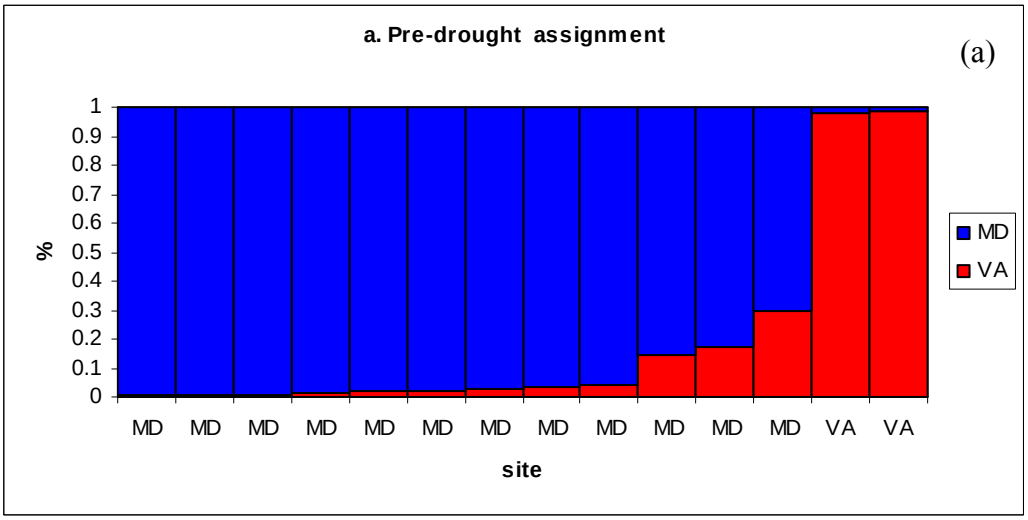

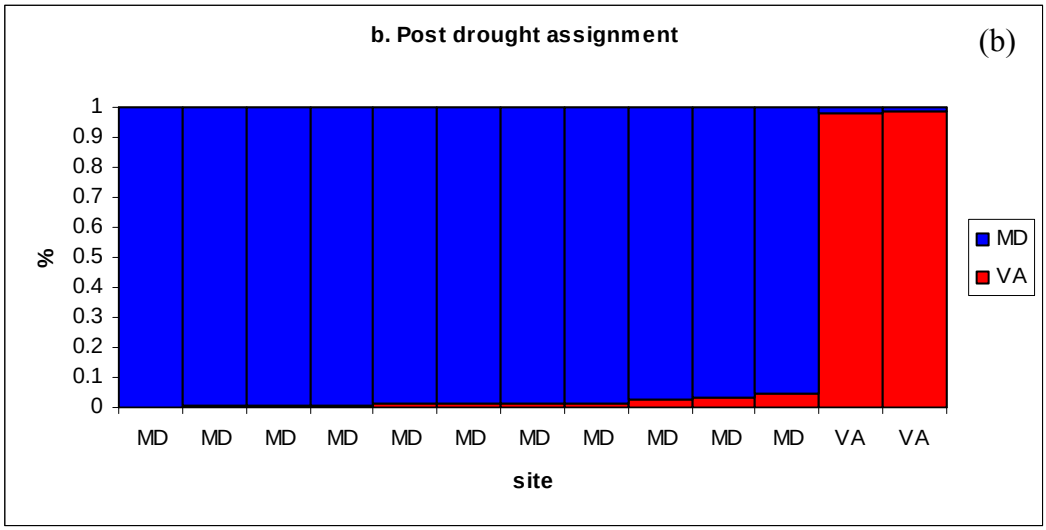

Figure 6. Changes in population structure at a recolonized site following the drought.

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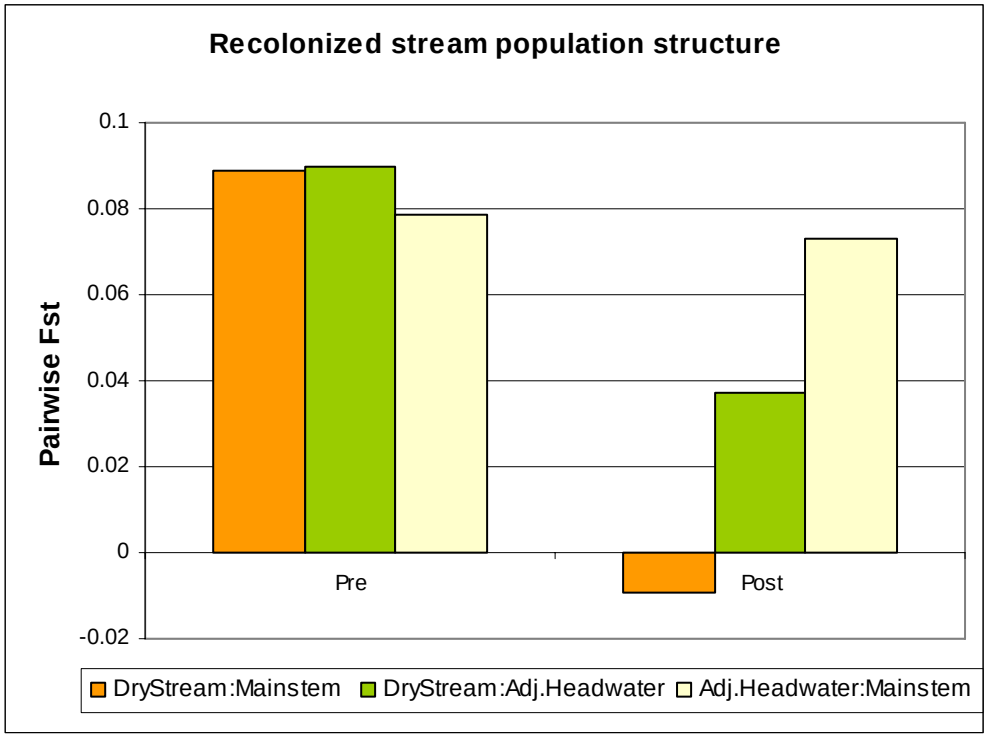

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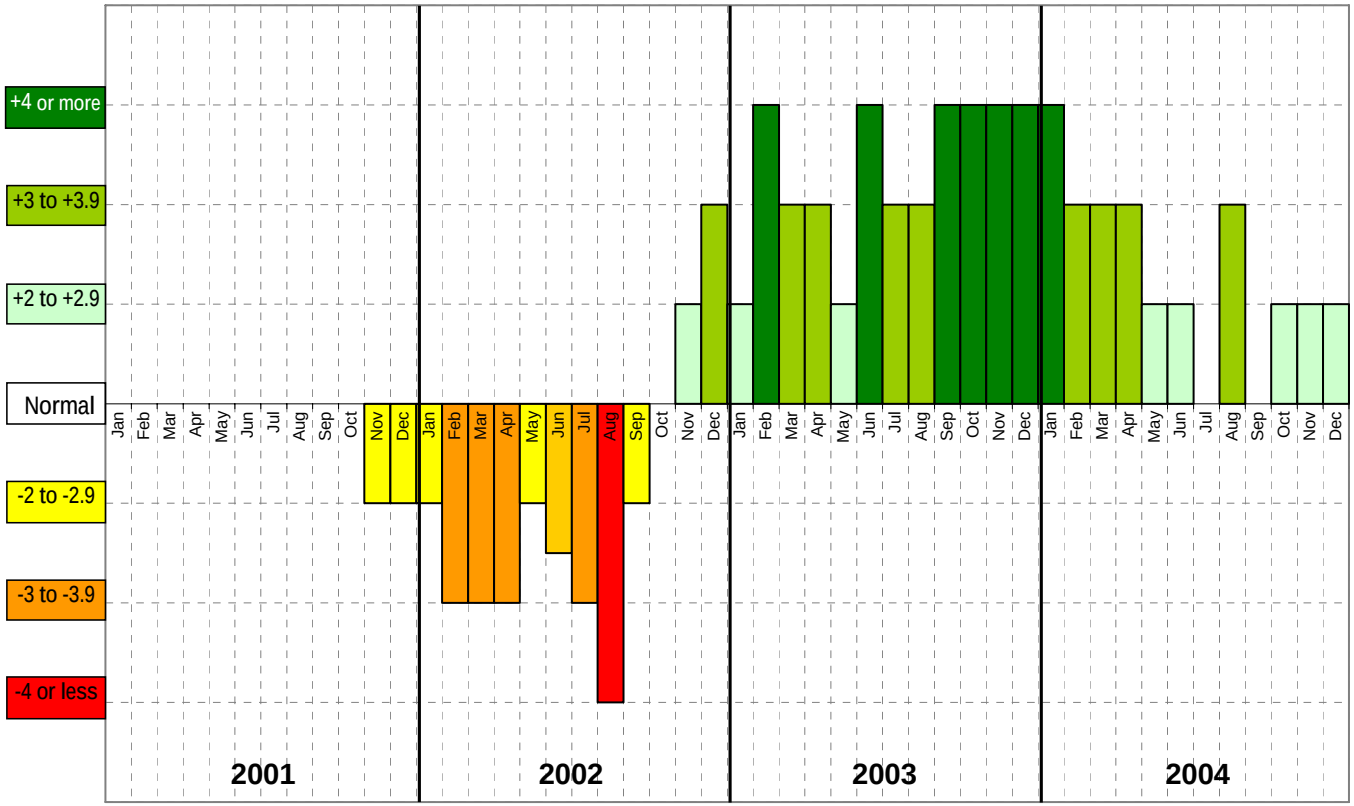

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