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ORIGINAL PAPER

Dispersal via stream corridors structures populations of the endangered St. Francis' satyr butterfly (*Neonympha mitchellii francisci*)

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Abstract Habitat fragmentation may reduce gene flow and population viability of rare species. We tested whether riparian corridors enhanced gene flow and if human habitat modification between riparian corridors subsequently reduced dispersal and gene flow of a wetland butterfly, the US federally endangered St. Francis' satyr butterfly (*Neonympha mitchellii francisci*). We surveyed nine populations throughout the taxon's range using five polymorphic microsatellite loci. We found that genetic diversity of *N. m. francisci* was relatively high despite its restricted distribution, and that there is little evidence of population bottlenecks or extensive inbreeding within populations. We found substantial gene flow and detectable first generation migration, suggesting that *N. m. francisci* is unlikely to be currently endangered by genetic factors. Pairwise population differentiation and clustering indicate some structuring between populations on different drainages and suggest that dispersal probably occurs mainly via a stepping stone from the closest riparian corridors. However, genetic differentiation between geographically close populations suggests that isolation by distance is not solely responsible for population structure, and that management actions should be targeted at maintaining connectivity of riparian and upland habitats.

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Introduction

The greatest threat to biodiversity, including to the diversity and population viability of butterflies, is the loss and fragmentation of native habitats (New 1991; Wilcove et al. 1998). The most popular landscape strategy for reducing the effects of habitat fragmentation is the conservation or restoration of landscape corridors (Hilty et al. 2006). Natural and artificial corridors increase dispersal, thus increasing gene flow (Hale et al. 2001; Mech and Hallett 2001), (re)colonization of patches, and population viability. Although corridors have been shown to be effective at increasing dispersal for butterflies in numerous studies (Sutcliffe and Thomas 1996; Haddad 1999; Haddad et al. 2003), there has been virtually no work to show that this increased movement has an impact on populations, and no work has shown that corridors impact populations of endangered species (Haddad and Tewksbury 2006). Here, we test the effects of naturally-occurring riparian corridors on the population structure of a US federally endangered butterfly species, the St. Francis' satyr (*Neonympha mitchellii francisci*).

One of the strongest methods available to test the effects of landscape factors like corridors on populations is through use of genetic data (Epps et al. 2007). The effects of the spatial arrangement of habitat and non-habitat on movement and gene flow at the landscape level are often revealed through population genetic structure (Hartl and Clark 1997; Hanski 1998; Lushai et al. 2000). Molecular genetic studies can provide valuable information about dispersal between small, extinction-prone local populations

and, especially when used in conjunction with ecological studies such as mark-release-recapture, can greatly enhance the management and recovery of threatened and endangered species and their habitat (Keyghobadi et al. 1999; Manel et al. 2003). Furthermore, genetic data can contribute significantly to the management and recovery of imperiled species by identifying populations with low genetic diversity that require focused management to prevent inbreeding and further loss of variability (Frankham et al. 2002). The delineation of subdivided populations can help guide the translocation of individuals to augment small, declining populations or the reintroduction of extirpated species (Storfer 1999; Bouzat et al. 2008). Application of genetic techniques to the study of butterflies has become particularly appealing as non-lethal methods have been developed to sample tissues, usually from small wing clippings (Chaline et al. 2004; Saastamoinen and Hanski 2008).

The endangered butterfly *Neonympha mitchellii francisci* is the ideal study organism to test the effects of landscape connectivity on population genetic structure. *N. m. francisci* are associated with dynamic, early stage wetlands located along streams. These wetlands are created by beaver impoundments that are subsequently abandoned (Bartel et al. 2010). The larval food plant of *N. m. francisci*, likely a sedge in the genus *Carex*, is among the early successional vegetation that emerges after a beaver impoundment is abandoned (Hall 1993; Hall and Haddad 2005). As the sedges and grasses succeed to shrubby and woody vegetation, the habitat quickly becomes unfavorable for *N. m. francisci* (Bartel et al. 2010). In some cases, fire can set back succession. Regardless, butterflies cannot persist through disturbance or through succession to riparian forest. Riparian habitats may serve as corridors connecting degrading subpopulations to early successional habitat. Although work has been done using mark-release-recapture studies to track dispersal of *N. m. francisci*, the butterfly is so sedentary that almost no dispersal has been detected, and genetic techniques are needed to determine the effects of riparian corridors on population structure (Kuefler et al. 2008, 2010).

In this paper we evaluated microsatellite diversity across nine subpopulations of *N. m. francisci* found in five different stream drainages. We used five newly developed microsatellite loci to test for differentiation across the range and to test how riparian corridors and other landscape factors (e.g. site size, isolation, habitat barriers) affect dispersal. In the process, we also (1) identified levels of genetic diversity and differentiation within and among populations (2) tested for evidence of recent bottlenecks, (3) estimated recent gene flow among populations, and (4) assimilated this information to provide specific conservation measures for genetically inferred populations and make recommendations for appropriate translocation and reintroduction strategies.

Materials and methods

Study species

Neonympha mitchellii francisci is endemic to small, isolated patches of specialized wetland habitat on the US. Army installation of Fort Bragg, NC, and is one of the rarest butterflies in North America (Hall 1993; Hall and Hoffman 1994). Loss of habitat (including beavers that maintain wetlands and were exterminated from North Carolina around 1900), desirability as a collectors' specimen, and previous fire suppression have resulted in the species' endangerment. Its conservation and restoration will only be possible after understanding of its population structure and demographic parameters, habitat requirements, movement patterns, and other aspects of its ecology and natural history (Hall 1993; Hall and Hoffman 1994; Kuefler et al. 2008). Significant advances have been made in understanding the distribution, host plant preferences, population size, demographic parameters, and movement of *N. m. francisci* (Hall 1993; Hall and Hoffman 1994; Haddad et al. 2008a; Kuefler et al. 2008; Bartel et al. 2010). Ft. Bragg specifically has supported efforts that have included long-term monitoring, observation, and evaluations of currently and potentially suitable habitat for the subspecies (Kuefler et al. 2010). What is still not understood is how landscape factors affect population structure for this butterfly.

Neonympha mitchellii francisci is generally found within the approximately 650 km^2 of the North Carolina Sandhills Region occupied by Fort Bragg. The landscape is uncharacteristically hilly compared to the surrounding Southeastern Coastal Plain ecoregion, and supports a preponderance of upland longleaf pine woodlands as well as the unique bottomland stream floodplain communities that *N. m. francisci* inhabits. The total area of habitat occupied by St. Francis' satyr is less than 50 ha. As with many other rare, temperate butterfly species, *N. m. francisci* broadly exists in a metapopulation population structure of environmentally stochastic local extinction mediated by colonization and recolonization by individuals from other subpopulations within the larger metapopulation (Hall 1993; Hall and Hoffman 1994). A key limit to understanding *N. m. francisci* population size and structure is that it occurs within and outside of artillery impact areas. The subpopulations within artillery impact areas are not regularly accessible to researchers. Therefore, a genetic analysis of the structure and subdivision of the colonies outside and within areas of restricted access is particularly

important to complement empirical and observational research that has been conducted in unrestricted sites.

Sample collection

The use of a small piece of wing has been employed as a non-lethal and minimally invasive procedure for sampling DNA from bees and butterflies (Chaline et al. 2004; Hamm et al. 2010). Several studies justify the benign nature of the technique as simulating unsuccessful predation attempts by birds that frequently result in functional butterflies that are missing parts of their wings (Chaline et al. 2004; Keyghobadi et al. 2006; Saastamoinen and Hanski 2008). Hamm et al. (2010) found that clipping a 3 mm^2 piece of the wing from the anal angle of the hindwing resulted in no significant detrimental effects on survival or activity level of *Satyrodes eurydice*, a butterfly in the same subfamily as *N. m. francisci*, and of similar size and shape. Employing this technique, we used fine-tipped forceps or scissors to clip an approximately 2 mm^2 piece of the metathoracic wing, being certain to include a section of the anal vein (containing DNA-rich hemolymph) in the sample. We collected wing clippings over four breeding periods in 2008 and 2009 (*N. m. francisci* are bivoltine) from a total of 90 individuals at nine sites along five stream drainages (Fig. 1). Precise sampling locations cannot be published and Fig. 1 is deliberately vague to protect the butterflies from the threat of illegal collection; however, sampling coordinates may be provided to credentialed researchers who demonstrate legitimate interest. Males were preferentially sampled to minimize any unforeseen impacts on these highly vulnerable populations. We were also able to collect an additional nine specimens through captive rearing of *N. m. francisci*. One adult female died (wing wear indicated that this was most likely due to advanced age) while in a chamber designed for oviposition and was immediately collected; eight other newly-emerged individuals from three different broods of *N. m. francisci* were reared in captivity and were sacrificed or wing-clipped. In the field, most samples were placed in 70% ethanol, whole individuals were placed on ice; all samples were stored at −80°C prior to DNA extraction.

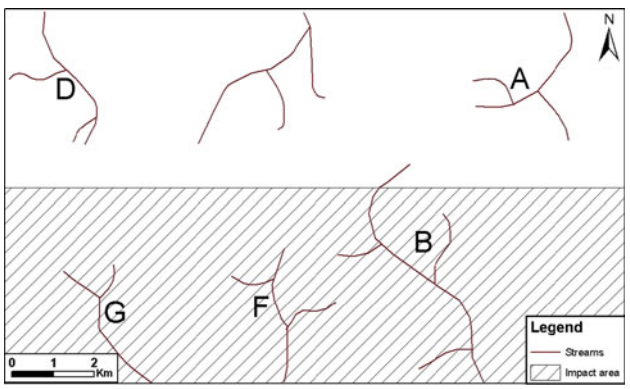

Fig. 1 Five sampled drainages on Fort Bragg, NC as in Table 1. Population sites cannot be published (data may be available from corresponding author upon request)

Microsatellite analysis and genotyping

We extracted total DNA from individual butterflies using the DNeasy tissue kit protocol (Qiagen, Valencia, CA). In Lepidopterans, and in insects in general, the general scarcity of microsatellites and a pattern of microsatellites clustering with others with like flanking regions can impede isolation and optimization of microsatellites and quickly consume small DNA samples (Zhang 2004; Meglecz et al. 2007). Our genomic DNA yield for some wing-clipped individuals was insufficient, in which case we used the REPLI-g whole genome amplification protocol (Qiagen, Valencia, CA) to increase our DNA yield up to 100× the original concentration. DNA was then serially enriched twice for microsatellites using 3 probe mixes (mix 2 = (AG)12, (TG)12, (AAC)6, (AAG)8, (AAT)12, (ACT)12, (ATC)8; mix 3 = (AAAC)6, (AAAG)6, (AATC)6, (AATG)6, (ACAG)6, (ACCT)6, (ACTC)6, (ACTG)6; mix 4 = (AAAT)8, (AACT)8, (AAGT)8, (ACAT)8, (AGAT)8) following Glenn and Schable (2005) with the alterations in protocol described in Lance et al. (2010).

Briefly, DNA was digested with restriction enzymes RsaI and BstUI (New England Biolabs) in separate reactions, pooled, and then ligated to double-stranded linkers. Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were then captured on magnetic streptavidin beads (Dynal). There were two primary changes to the Glenn and Schable (2005) protocol. First, we used different linkers: SimpleX-1 (Flanagan et al. 2010) Forward 5′-AAAACGTCGTGCGGAATC and SimpleX-1 Reverse 5′-GATTCCGCACGACG (Henningsen and Lance 2010). Second, the enriched libraries were sequenced on a 454 using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, a Roche company, Branford, CT). Sequences were subjected to a 3′ quality trim where only one base in the last 25 bases of the sequence contains a quality score less than 20 or alternatively contains one ambiguous base. CAP3 (Huang and Madan 1999) was then used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. Along with singlets, contigs of two or three sequences were searched for the presence of microsatellite DNA loci using the program MSATCOMMANDER version 0.8.1 (Faircloth 2008) and primers designed with Primer3. One primer from each pair was modified on the 5′ end with

an engineered sequence (CAG tag 5′-AGTCGGGCGTC ATCA-3′) to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled for detection. All methods for sequencing, microsatellite identification, primer design, and primer screening are as described in Lance et al. (2010), Flanagan et al. (2010) and Henningsen and Lance (2010).

Ninety-six primer pairs were tested for amplification and polymorphism using DNA obtained from eight individual *N. m. francisci*. PCR amplifications were performed in a 12.5 µl volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04 µM tag labeled primer, 0.36 µM universal dye-labeled primer, 3.0 mM MgCl2, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 55 and 45°C were used for all *N. m. francisci* loci. Touchdown cycling parameters consisted of 20 cycles of 96°C for 30 s, highest annealing temperature of 55°C (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in deWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Five of the tested primer pairs amplified high quality PCR product that exhibited polymorphism ("Appendix"). After Bonferroni correction for multiple comparisons, three *N. m. francisci* loci showed significant deviations from expectations under Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) was detected for none of the loci out of 10 paired loci comparisons. The deviations from HWE were expected given that our samples came from an extremely geographically restricted subset of individuals. The five loci from *N. m. francisci* were also successfully screened across ten individuals of its federally endangered sister species, *N. m. mitchellii*, from samples obtained in the Midwest and Alabama. Due to the much wider distribution of *N. m. mitchellii*, we expect a concomitant decrease in deviation from HWE across these five loci.

Analysis of genetic diversity

Genetic diversity was first calculated across the whole dataset as the average number of alleles per locus (A), expected heterozygosity (H_E) and observed heterozygosity (H_O) using GENEPOP v. 4.0 (Rousset 2008). Tests for deviations from HWE and for linkage disequilibrium also were conducted using GENEPOP v4.0 (Rousset 2008). The FSTAT program v 2.9.3.2 (Goudet 2002) was used to determine the inbreeding coefficient (F_{IS}) and allelic richness (A_R) for each population.

We used BOTTLENECK (Cornuet and Luikart 1997), an analysis that requires a minimum sample size of ten individuals, to test for recent reductions in population size and signatures of past bottleneck events by drainage. BOTTLENECK tests for the relative heterozygosity excess that is apparent for a few generations after a bottleneck. This excess of heterozygosity develops because the loss of rare alleles leads to a much faster decline of the allelic diversity than of the level of heterozygosity. The two-phased model (TPM) of mutation (DiRienzo et al. 1994) was used following the settings recommended by the authors. We used Wilcoxon's sign rank test across all loci within a population to evaluate whether a significant excess of heterozygosity exists.

We used assignment tests to determine the proportion of individuals that migrated to a population and to identify which of these individuals were first generation migrants. Assignments were carried out in GENECLASS 2 (Piry et al. 2004) using the resampling algorithm of Paetkau et al. (2004), which is based on the 'L_home' likelihood estimation (to account for not sampling all possible source populations) and with the probability of assignment based on a threshold of $P < 0.05$ and on 10,000 simulated individuals.

Genetic differentiation and population structure

Analysis of molecular variance (AMOVA) and pairwise F_{ST} were calculated as measures of population genetic differentiation in ARLEQUIN version 3.1.1 (Excoffier et al. 2005). We determined the relationship between pairwise genetic and geographic distances using a Mantel test of isolation-by-distance. Estimations among population pairs were calculated with 1,000 iterations in GENEPOP (Raymond and Rousset 1995), using the natural log of the Euclidean distance in meters as the geographic distance and $F_{ST}/(1 - F_{ST})$ as the genetic distance.

We also used a Bayesian assignment approach, implemented in the program STRUCTURE 2.2, to assign individuals to clusters by determining the K that minimize deviations from HWE and LD (Pritchard et al. 2000). We conducted 20 replicate runs for values of K ranging from 1 to 10, and five replicate runs for values of K ranging from 11 to 20. For all analyses of K, the Markov chain Monte Carlo (MCMC) was run for 1,000,000 simulations with a burn-in period of 100,000 simulations. We used the admixture model with correlated allele frequencies for each potential value of K, as suggested by Falush et al. (2003). The best estimate of K was determined using the method of Evanno et al. (2005), which uses the maximum change in

the log probability of the data for successive values of K. For the best estimate of individual assignments, membership coefficients were averaged over the 20 replicate runs for the optimal K value, using the "Greedy" algorithm and 100 permutations of the data in the program CLUMPP (Jakobson and Rosenberg 2007). DISTRUCT 1.1 (Rosenberg 2004) was then used to visualize the estimated membership coefficients outputted from CLUMPP.

Results

Overall genetic diversity

For the five polymorphic loci surveyed, a total of 53 alleles were observed among 99 individual *N. m. francisci*. Seven to 14 alleles were found at each locus, however one locus, NEMI28, was monomorphic in two populations. Six out of the nine populations (66%) significantly deviated from expected Hardy–Weinberg proportions at single loci after Bonferroni correction (Table 1). Although deviation from Hardy–Weinberg equilibrium may indicate the presence of null alleles, it also may be due to the historically small metapopulation size, a failure to detect rare alleles due to a low sample size, or sampling errors such as the Wahlund effect that predict a significantly lower observed than expected heterozygosity if members of subdivided populations are mistakenly grouped into a single population (Allendorf and Luikart 2007).

No linkage disequilibrium was observed between pairs of loci across populations. Overall observed heterozygosity ($H_O = 0.462$) was somewhat lower than expected heterozygosity ($H_E = 0.605$) and this result coincided with a Hardy–Weinberg global test that revealed significant deviation from HWE for two populations ($P < 0.05$) within the 'D' drainage and the 'A' drainage. Allelic variation, expressed by the average number of alleles per locus (A_N) and allelic richness (A_R) averaged 10.6 and 3.5, respectively and was fairly consistent overall. The highest genetic diversity ($A = 5.0$, $A_R = 4.3$) was found in the 'F' drainage and the lowest genetic diversity ($A = 3.6$, $A_R = 2.91$) was found in population 1 of the 'D' drainage, in a population that had recently undergone a precipitous decline (Lessig et al. 2010). Overall, the effect of inbreeding was relatively minimal, with F_{IS} estimates ranging from −0.277 to 0.222, though the highest value of F_{IS} was found in populations 1 and 2 in the 'D' drainage. Analysis with the software BOTTLENECK showed that population 1 in the 'D' drainage also gave marginally significant evidence of a recent bottleneck ($P = 0.078$); none of the other populations showed evidence of recent bottlenecks.

Population structure and migration

The overall mean F_{ST} value was 0.078 and pairwise estimates ranged from $F_{ST} = -0.010$ to 0.204. Significant population differentiation ($P < 0.001$) was found for slightly less than half (17 out of 36) of the population pairs after Bonferroni correction, particularly between the 'D' and 'A' drainages and the rest of the study area. Though non-significant, there was a trend toward linear isolation by distance (Fig. 2; $P = 0.080$). A Mantel test that incorporated landscape connectivity into our distance measurement as opposed to simple Euclidian space, might have revealed a relationship between genetic and geographical distance (Hanski 1998).

The results of the population assignment conducted by GENECLASS identified five potential F_0 migrants, and indicated that first generation migration most often occurred in the central part of the study area, between and within the 'F' and 'B' drainages, with potentially infrequent migration occurring within the two populations along the 'D' drainage (Table 2). The one population, 'D', that may have recently undergone the bottleneck did not show evidence of having recently received an immigrant from

Table 1 Mean number of alleles (A_N), number of polymorphic loci (P) observed heterozygosity (H_O) and expected heterozygosity (H_E), average effective number of alleles (A_E), and F_{IS} value and F-value by sample population on Fort Bragg, NC

Sample population	Drainage	N	A_N	A_E	P	H_O	H_E	F_{IS}	F
1	D	22	5.0	2.6	100.0	0.482	0.566	0.222	0.4
2		7	3.6	2.5	100.0	0.420	0.518	0.198	0.4
3	A	12	4.4	2.9	100.0	0.494	0.594	0.138	0.47
4	B	12	5.2	3.0	80.0	0.452	.0571	0.178	0.67
5		7	3.8	2.3	100.0	0.371	0.494	0.185	0.57
6	F	4	2.6	2.0	100.0	0.417	0.431	−0.277	0.59
7		11	4.2	2.9	80.0	0.455	0.553	0.114	0.41
8		10	5.4	2.7	100.0	0.560	0.575	0.083	0.38
9	G	14	4.8	2.6	80.0	0.423	0.507	0.068	0.39

Table 2 Dispersers identified in populations including the individual identifier (ID), sampling locality, probability of the individual being a disperser, and the most likely population of origin as assigned by GENECLASS2

ID	Locality	Disperser probability	Origin (probability)
23	1	0.993	2 (0.491)
49	5	0.991	4 (0.799)
65	7	1.000	8 (0.712)
68	7	0.999	8 (0.587)
75	8	0.995	5 (0.697)

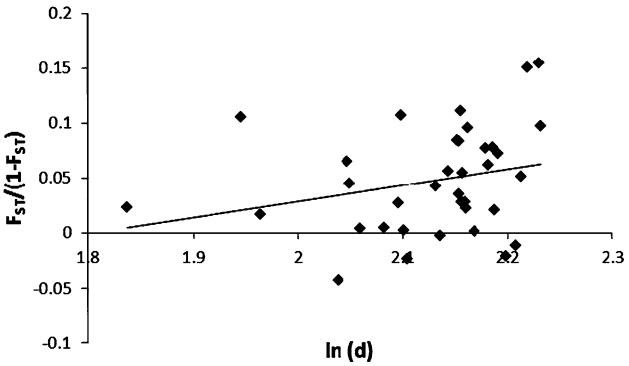

Fig. 2 Mantel test of isolation-by-distance correlation, $F_{ST}/(1 - F_{ST}) = 0.1459(\ln(\text{geographic distance, m})) - 0.263$, $R^2 = 0.0664$, $P = 0.080$

outside the drainage. Historic gene flow among populations was estimated by the average frequency of alleles that are unique to individual populations using Slatkin's (1985) private alleles method and yielded an estimate of $N_m = 1.85$. An indirect method of estimating gene flow using the formula $N_m = (1 - F_{ST})/4F_{ST}$ (Wright 1951) yielded a similar $N_m = 2.94$. As evidenced by numerous non-significant pairwise F_{ST} values between the 'G', 'F' and 'B' drainages (Table 3), most of this historic gene flow appears to be accounted for within the central part of the study area.

The mean log likelihood from replicate runs in STRUCTURE was used to determine the best value of K using the method of Evanno et al. (2005). The STRUCTURE analysis tested for the presence of landscape barriers that disrupt movement between adjacent corridors. This analysis suggested the existence of three main clusters (Fig. 3), as shown by the modal value of ΔK (Fig. 4), but also confirms the existence of relatively high gene flow throughout the metapopulation. Cluster 1 consisted of individuals from 'D' and 'G' drainages,with all individuals from population 2 assigned to Cluster 1 with high confidence and an average estimated membership coefficient of 0.789. Individuals from population 1 in 'D' drainage and population 9 in 'G' drainage were assigned to Cluster 1 with an average estimated membership coefficient of 0.621. Cluster 2 contained the 'B' and 'F' drainages with the highest level of admixture and the lowest membership coefficient of 0.432. Cluster 3 consisted of individuals in the 'A' drainage with high confidence and a membership coefficient of 0.776. An AMOVA of hierarchical gene diversity using drainages to group populations revealed that most (80.36%) of the variance in allele frequencies was accounted for within individuals, 11.57% occurs among individuals within populations, 3.48% is due to differences among populations within drainages, and 4.59% of the variation is due to differences among drainages. Additional AMOVAs using the STRUCTURE clusters as groups were very similar to the drainage results, with the three groups identified by STRUCTURE accounting for 5.99% of the total variation in the data set. The global fixation indices were 0.126, 0.153, 0.196 for

Table 3 Pairwise F_{ST} values for nine populations of *Neonympha mitchellii francisci*

Drainage	D		A	B		F			G
Pop.	1	2	3	4	5	6	7	8	9
1	0.000								
2	0.007	0.000							
3	**0.187**	**0.204**	0.000						
4	0.033	0.061	**0.128**	0.000					
5	**0.061**	0.001	**0.193**	0.024	0.000				
6	0.044	**0.185**	0.136	0.033	**0.118**	0.000			
7	**0.058**	**0.155**	**0.132**	−0.010	−0.015	0.058	0.000		
8	**0.119**	**0.084**	**0.101**	0.046	0.062	0.007	0.008	0.000	
9	0.025	0.002	**0.161**	0.001	**0.111**	0.041	**0.047**	**0.098**	0.000

Bold values are statistically significant following Bonferroni corrections, $\alpha \leq 0.0001$

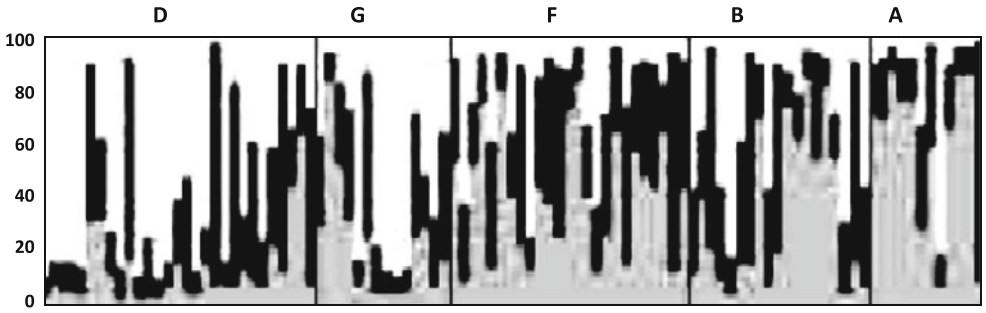

Fig. 3 Histograms of averaged assignment probabilities (clustering analysis calculated by STRUCTURE, averaged by CLUMPP and visualized by DISTRUCT). Each *vertical bar* represents an individual and its assignment proportion into one of the three clusters. Individuals are arranged in order by drainage, and drainages are labeled from west to east (as seen in Fig. 1)

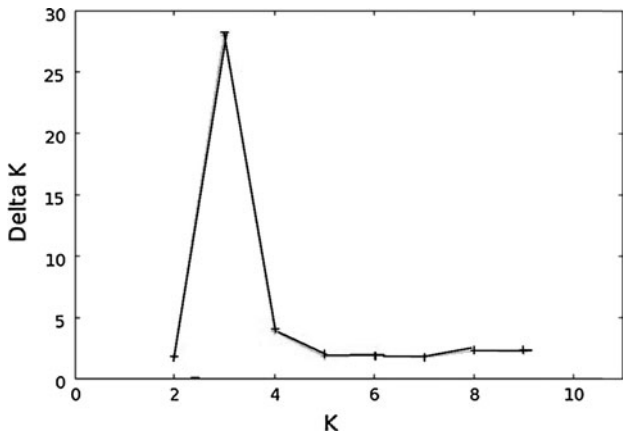

Fig. 4 ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$. The modal value of this distribution is the true value of K^*, or the uppermost number of clusters. In this case it is three clusters

F_{IS}, F_{ST}, and F_{IT}, respectively. F_{SC} and F_{CT} were 0.037 and 0.046.

Discussion

Despite the relatively sedentary behavior *of N. m. francisci* observed in mark-recapture and behavioral studies (Kuefler et al. 2008, 2010), there is a relatively high level of gene flow and demonstrable movement between populations along adjacent riparian corridors. Consistent with the study conducted by Haddad (1999), the results of our clustering assignments suggest that *N. m. francisci* is more likely to utilize the closest riparian corridors to disperse between habitats. There is also a positive association between levels of gene flow, genetic variation, and landscape connectivity that is probably the result of higher population densities of butterflies in patches connected by corridors (Haddad and Baum 1999). Furthermore, landscape connectivity between stream drainages, defined by lack of roads and non-forest openings, also appears to facilitate gene flow. Thus, identifying and preserving dispersal corridors may help to maintain the balance between local extinctions and colonizations within the metapopulation structure of *N. m. francisci*.

Genetic variability

In spite of moderately high levels of variability overall ($H_E = 0.602$) and across microsatellite loci by population (Table 1), we were able to identify landscape factors structuring the *N. m. francisci* metapopulation on Fort Bragg. Congruent estimates from different analyses reveal that the overall level of genetic differentiation was relatively low ($F_{ST} = 0.078$), albeit significant between almost half of the pairwise estimates. Estimates of gene flow based on F_{ST} ($N_m = 2.94$) and on the mean frequency of private alleles ($N_m = 1.85$) are similar, despite different theoretical bases, and indicate that historical gene flow among populations has been sufficient to prevent substantial differentiation across the entire range of *N. m. francisci* due to genetic drift. Although the current population sizes and total areas of the sites inside the impact areas are unknown, results of population surveys using transect counts suggest that the three populations on Drainages 'D' and 'A' are likely to be small relative to those within the impact areas (Hall and Hoffman 1994). The long-term survival of populations outside impact areas remains tenuous because their small sizes make them highly vulnerable to extinction due to environmental and demographic stochasticity, and their isolation hinders rescue from extinction by individuals that could colonize from other populations. Moreover, population genetic theory predicts that smaller populations are more likely to suffer the deleterious effects of genetic drift and inbreeding and show lower levels of genetic variability than large populations (Nei 1973; Hartl and Clark 1997; Frankham et al. 2002).

Population structure and dispersal

Our results suggest that dispersal between drainages occurs primarily between the nearest riparian corridors, including between G and D, and between F and B. Populations 1 and 2 on the 'D' drainage are clustered with population 9 on the 'G' drainage. Although these populations are not the closest geographically, they are closest and most accessible drainages when measured via riparian corridors. Observational evidence supports our genetic evidence that *N. m. francisci* utilizes riparian corridors for dispersal. Population 1 on Drainage 'D', the most isolated drainage, was recolonized within a year subsequent to the dismantling of a beaver dam that had partially inundated the site (Haddad et al. 2008b). Additionally, a thriving colony was found on a nearby drainage close to a population (unsampled due to extremely low population density) that was recently extirpated due to flooding caused by beavers (Haddad et al. 2008b; Lessig et al. 2010).

A significant level of differentiation exists between peripheral and central populations. Gene flow between adjacent drainages appears to occur most frequently via occasional episodes of stepping-stone dispersal. First-generation migrants were primarily restricted to the central part of the study area in Drainages 'F' and 'B', whereas peripheral populations had lower probability of receiving first-generation migrants and are thus less connected to the metapopulation. This finding suggests a source-sink model, where genetic variation is expected to be much higher in the center of the habitat than at the edges (Wilkins and Wakeley 2002). A positive relationship clearly exists between the presence of *Carex* sedges and butterfly population presence and size, and once a wetland has succeeded beyond the stage where the sedges are dominant, the abundance of the butterfly declines sharply. For a subspecies such as *N. m. francisci* that specializes on habitat that is dependent on periodic disturbance, we recommend that effort be devoted to the maintenance of habitat in a variety of locations to facilitate colonization and recolonization that will sustain healthy levels of genetic variability within the metapopulation structure of *N. m. francisci* on Fort Bragg.

Genetic differentiation between populations that are geographically close suggest that landscape barriers that fragment corridors between different drainages, including artillery ranges and paved roads, potentially impede migration. The three genetic clusters revealed by STRUCTURE generally support the pairwise population differentiation between the different drainages, and also suggest there is a barrier to gene flow between the 'G' drainage that prevents it from becoming completely admixed with drainages 'B' and 'F'. A number of artillery ranges partially extend along approximately half the length of the 'G' drainage, running along both sides of a paved road, and may be impeding the dispersal of butterflies between these two drainages. A paved road that separates the populations on the 'B' and 'A' drainages could explain the strong differentiation between these otherwise geographically close populations. This contrasts with the lack of differentiation found for populations on drainages 'B' and 'F' that are separated by high quality intervening habitat. An alternative explanation is that there are significant differences in habitat that have resulted in local adaptation between drainages that are relatively close together.

One potential shortcoming of our study is that, due to restricted access to most of the impact area, we were unable to thoroughly sample all populations along every drainage. Peterson and Denno (1998) have suggested that finer-scale sampling (e.g. along drainages within the impact area) may reveal a strong signal of isolation-by-distance among many sedentary phytophagous insects. Very high levels of gene flow over the entire distribution of the butterflies could explain the non-significant result; however, the differentiation between drainages refutes this explanation. Populations may not be at equilibrium with respect to conflicting forces of gene flow and genetic drift as a result of an historical event, but the lack of evidence for a genetic bottleneck indicates otherwise. A least-cost path analysis using remote sensing tools to calculate a matrix of effective separation distances to replace the geographical distance matrix in the Mantel test could be informative. If the correlation improves over the purely spatial analysis, we could potentially conclude that additional landscape factors are influencing the dispersal of the butterflies (Stevens et al. 2006).

Management recommendations

A key aspect of the management of *N. m. francisci* may be the supplementation of waning populations or recently restored habitats with captively reared individuals. Our results support the hypothesis that some of the remaining populations in small and isolated fragments might be threatened with extirpation. The significant relationship observed by Kuefler et al. (2010) between site area and population size indicates that small sites are less likely to support viable populations of *N. m. francisci* than the larger sites in the center of the study area. The small, peripheral populations on drainage 'D' also contained less genetic variation than the larger, more closely connected interior populations and showed marginally significant signs of a recent genetic bottleneck. Although the observed microsatellite variation is expected to be selectively neutral, its loss may indicate a correlated loss of potentially adaptive genetic variation and a subsequent decrease in fitness.

When populations have diminished genetic diversity and a lower level of gene flow, introduction of captively reared individuals may be the best means of ensuring long term population viability with the current landscape configuration. From a genetic standpoint, there is sufficient gene flow throughout the entire metapopulation to allay concerns about local adaptation and outbreeding depression. Furthermore, captively reared individuals that were experimentally introduced into appropriate habitat during the 2009 and 2010 breeding seasons have survived and successfully reproduced, at least for short time periods (Lessig et al. 2010).

It is critically important that the populations of *N. m. francisci* outside the artillery impact areas are protected from anthropogenic influences. The various sub-populations across the subspecies' range are distant enough to experience different stochastic effects, but still permit dispersal between them. For this reason, the extirpation of a single sub-population is not an irrevocable disaster, as individuals from another sub-population are able to repopulate the site, either naturally or with human assistance. However, the total number of existing sub-populations is very small, and simultaneous harm to multiple subpopulations could be disastrous to the subspecies persistence. By protecting populations outside the impact areas, we will maintain a potential reserve of colonists if an environmental catastrophe were to deplete the population within the impact area. We will also continue to build our knowledge base of these perilously endangered butterflies so we can pinpoint ecological functions and target our management efforts with even greater precision in the future, particularly as we acquire additional information about spatial variation in site areas and isolations.

Riparian corridors, as well as intact upland habitat between riparian areas, have been shown to facilitate movement of stream dwelling animals (Grant et al. 2010). These dispersal pathways are vital to the persistance of *N. m. francisci* metapopulation structure and it is critically important that potential landscape barriers are removed to ensure unrestricted movement. Furthermore, we recommend preserving and restoring high-quality habitat in a variety of locations to protect the source-sink dynamics that sustain the metapopulation structure of *N. m. francisci* on Fort Bragg.

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Appendix

See Table 4.

Table 4 Details for five polymorphic microsatellite loci developed for *Neonympha mitchellii francisci*

Locus	Primer sequence 5′ → 3′	Repeat motif	Size (bp)	N	*k*	H_o	H_e	PI
Nemi26	F:*GGTCAGAAGAGGGTATTGGA R: CAGGTTGCTTCTCACGCATT	$(ATCT)_{14}$	125–181	23	6	0.348†	0.716	0.130
Nemi 28	F:*GTCTCTAGACCCAGCGGTT R: ACAAAGTCGATCTGGCTGAA	$(CAGT)_{11}$	335–379	23	4	0.261	0.303	0.505
Nemi 59	F:*AATCGGTCAAGCCATTC R: AGGCTCGGATCAAGGTTTCT	$(ACAT)_{10}$	120–160	23	6	0.333†	0.549	0.233
Nemi62	F:*CCGACTACATCATAAGAAGGG R:TCTCTTTACCCACCACCGAA	$(ATGT)_{11}$	54–98	24	6	0.870	0.752	0.102
Nemi 67	F:*GCTACTTTGCACTTATGTTGCC R:AGTCTCTAGACCCAGCGGTT	$(ACTG)_{17}$	140–208	23	4	0.522†	0.499	0.294

The number of individuals genotyped is *N*; size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; *k* is number of alleles observed; H_o and H_e are observed and expected heterozygosity, respectively; PI is the probability of identity for each locus

Asterisk indicates CAG tag (5′-CAGTCGGGCGTCATCA-3′) label

† Significant deviations from Hardy–Weinberg expectations after Bonferroni corrections

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