Molecular Ecology (2006) **15**, 3553–3566 doi: 10.1111/j.1365-294X.2006.03034.x

Spatial population genetic structure and limited dispersal in a Rocky Mountain alpine stream insect

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Abstract

Using the mitochondrial cytochrome oxidase I (COI) gene, we assessed the phylogeographic structure of *Prosimulium neomacropyga*, a black fly (Simuliidae) whose distribution in the US Southern Rockies ecoregion is limited to alpine tundra streams. Given high habitat specificity, lack of hydrological connection between streams, and a terrestrial environment restrictive to insect flight, we hypothesized limited gene flow. A spatially nested sampling design showed that grouping populations according to high-elevation 'islands' of alpine tundra (which typically include headwater streams of > 1 watershed) explained a significant proportion of genetic variation while grouping streams according to major watershed (across islands) did not. Nested clade analysis and isolation-by-distance (IBD) relationships further implicated limited ongoing gene flow within but not among the isolated alpine islands. IBD was strong among five streams within an individual island using each of four alternative models of pairwise landscape connectivity for flying insects. Results of all landscape models were positively correlated, suggesting that straight-line distance is an acceptable surrogate for presumably more biologically meaningful connectivity measures in this system. IBD was significantly weaker across the entire study area, comprised of three separate islands. Overall, population structure was significant with F_{ST} = 0.38, suggesting limited dispersal across a small spatial extent.

Keywords: alpine streams, isolation by distance, mtDNA, population structure, Simuliidae, SSCP

Received 19 March 2006; revision received 21 April 2006; accepted 22 May 2006

Introduction

Movement of animals across landscapes is an important but oft-overlooked process influencing population (e.g. Wright 1940; Macdonald & Johnson 2001) and community (e.g. MacArthur & Wilson 1967; Palmer *et al*. 1996; Hubbell 2001) dynamics. Understanding animal movement is therefore an important goal for both basic ecology and conservation biology. However, two important obstacles to reaching this goal have been (i) landscapes are naturally heterogeneous, different elements having different and sometimes unpredictable effects on movement (Moilanen & Hanski 1998; Roland *et al*. 2000; Wiens 2001); and (ii) animal movement patterns are difficult to quantify by direct observation, especially across long distances (Slatkin 1985; Turchin 1998). If the movement of organisms across the landscape also results in gene flow, however, then analysis of spatial population genetic structure can be a robust method to address both these issues (Bohonak 1999). Relative levels of dispersal within and among spatially structured groups of populations can be compared using selectively neutral genetic markers, and components of the landscape that impede dispersal can be identified using phylogeographic principles (cf. Avise 2000).

Stream-dwelling species present an ideal opportunity for assessing spatial genetic structure because streams are clearly defined habitat patches embedded in an uninhabitable terrestrial matrix. Distinct populations are therefore easy to identify in this spatial context. Many stream species are insects that spend the majority of their lives as fully aquatic juveniles and then emerge as mature, often winged

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adults that mate and lay eggs back into a stream. Studies of population genetic structure of stream insects have suggested that adult flight dispersal between drainages is relatively common based on a lack of broad-scale genetic differentiation (e.g. Schmidt *et al*. 1995; Bunn & Hughes 1997; Hughes *et al*. 1998, 2000; Baker *et al*. 2003). Exceptions to this generalization have been found in some mountain stream insect species, where dispersal may be limited between watersheds due to steep drainage divides (Hughes *et al*. 1999, 2003b; Wishart & Hughes 2001, 2003; Monaghan *et al*. 2002), and further population differentiation may arise due to specialization on a rare and stable instream habitat type thereby increasing the risk associated with dispersal (Wishart & Hughes 2001, 2003; see also Roff 1990). We therefore may expect lower levels of gene flow among stream insect populations when (i) there are strong dispersal barriers between them, and/or (ii) the species of interest occupies a larval habitat that is spatially rare along the stream continuum.

In the highest-altitude areas of the Southern Rockies ecoregion of the USA (Omernik 1987), streams originate in the alpine ecological zone, which occurs above the permanent treeline [> *c.* 3300 m above sea level (a.s.l.) in northern Colorado]. Alpine streams in this area have a distinct assemblage of insect species, some of which are confined to these highest-elevation reaches (Allan 1975; Ward 1994; Finn & Poff 2005). It is likely that these alpine specialists were more widespread during Pleistocene glaciations (*c.* 10 000–100 000 years ago) when the spatial extent of the alpine zone would have been much more extensive than it is at present; the zone consists now only of remnant 'islands' of alpine tundra in a 'sea' of trees (Elias 1996; DeChaine & Martin 2004). For some terrestrial insect species, remnant alpine populations have a high degree of genetic structure that suggests isolation among alpine islands (e.g. Knowles 2001; DeChaine & Martin 2004). Such analyses have not been done on alpine stream insects, which provide a particularly interesting case in that even streams occupying a single alpine island will often occupy different major watersheds, the boundaries of which have been shown in many cases to be important dispersal barriers (e.g. Hughes *et al*. 1999; others cited above).

An important feature of alpine areas is the extreme spatial heterogeneity in terrestrial microclimates, driven by high topographic relief (Bowman 2001). Such heterogeneity may have significant effects on dispersing animals, such that the straight-line distance between two points is not likely to be a biologically realistic route. Several studies outside of the alpine zone have used high resolution geographic information systems (GIS) to model important landscape features and predict animal movement (e.g. Schippers *et al*. 1996; Moilanen & Hanski 1998). More recently, similar models have been used in conjunction with spatial population genetic patterns to address various questions about dispersal pathways (Michels *et al*. 2001; Coulon *et al*. 2004; Spear *et al*. 2005). A key assumption of these studies is that physically closer populations will exchange migrants more often than distant ones; therefore, there will be a positive correlation between genetic distance and physical distance. This pattern is referred to as genetic isolation by distance (IBD), and the more biologically realistic the measure of physical distance, the tighter the expected fit between these two variables.

For stream insects, some studies have tested for IBD using both straight-line and stream-course distances to reveal whether adult vs. larval dispersal is more important in generating genetic patterns (e.g. Schultheis *et al*. 2002; Hughes *et al*. 2003b); however, to date none have attempted to test more biologically realistic measures of flight dispersal across the terrestrial landscape. The rapidly developing field of population genetics in stream ecology (e.g. Bunn & Hughes 1997; Monaghan *et al*. 2002; Schultheis *et al*. 2002; Wilcock *et al*. 2003) is increasing our understanding of broad-scale influences on insect distribution, and testing alternative models of landscape connectivity is an essential next step.

For this study, we addressed several questions regarding gene flow and population genetic structure of alpine stream insects using a representative black fly species (Simuliidae: *Prosimulium neomacropyga* Peterson) that occurs only in streams well above the treeline in the Southern Rockies (Adler *et al*. 2004). Within these environmental limits, *P. neomacropyga* is relatively common, occupying several headwater streams on multiple alpine islands in our study area. However, given potentially strong dispersal barriers between populations in the form of mountainous topography, in addition to the confinement of this species to a rare habitat type (the uppermost extent of the stream continuum), we hypothesized that gene flow would be limited.

Beyond assessing the overall spatial genetic structure, we asked whether the most important dispersal barriers were major drainage divides (as suggested in previous genetic studies of mountain stream insects) or lowlands separating islands of alpine tundra (as suggested in studies of alpine terrestrial insects). Further, at the finer scale of a single alpine island containing multiple headwater populations of *P. neomacropyga*, we developed several biologically realistic alternative models of dispersal connectivity given various elements of the terrestrial landscape. We then confronted each model with genetic data to assess which terrestrial features were most influential in determining spatial population structure, given an IBD assumption.

Because watershed divides have been shown to limit gene flow in stream insects, we predicted that the particularly steep and high-elevation divides in our study region would be important dispersal barriers at the within-island

scale. However, since *P. neomacropyga* is limited to the highest headwater streams, we hypothesized that there would be limited gene flow among alpine islands as well, even between streams on different islands located within the same major watershed.

Materials and methods

Study organism

Prosimulium neomacropyga is distributed extensively in Alaska and the Yukon and occurs only in small and sparsely distributed patches of alpine tundra to the south. The Southern Rockies of Colorado house the southernmost known populations, with nearest neighbours occupying the Beartooth Range of northern Wyoming (Adler *et al*. 2004), *c*. 300 km distant. A novel Y-chromosome sequence observed in larvae from Colorado (Adler *et al*. 2004) suggests their long-term reproductive isolation from other conspecifics.

Prosimulium neomacropyga is unusual among black flies in that it is obligately autogenous (i.e. mouthparts are incapable of piercing flesh; females cannot take a blood-meal for egg maturation). Long-distance flights in search of blood are thus unnecessary, and plant nectar may be obtained by adults as an energy source. The species is univoltine, overwintering as eggs and emerging as winged adults from mid-August to early September in Colorado. This narrow window for adult emergence and breeding probably precludes temporal reproductive isolation among these populations (see West & Black 1998). Little is known about mating behaviour; therefore, little has also been presumed about between-stream dispersal related to mating. This species is, however, closely related to other alpine/arctic species within the *P. macropyga* group, many of which have evolved reproductive strategies that avoid leaving the vicinity of the natal stream presumably due to the harsh terrestrial environment (Downes 1965).

Study sites and data collection

We collected *P. neomacropyga* from 11 alpine streams just east of the continental divide in the Rocky Mountain National Park (RMNP) area, Colorado (Fig. 1a). The alpine zone here extends from *c*. 3300–4200 m a.s.l., and much of the area below treeline is coniferous forest, with *Pinus*, *Picea*, *Abies*, and *Pseudotsuga* species dominant. Altitudinal variation among sites harbouring *P. neomacropyga* is minimal, ranging from 3450 to 3550 m, as this species appears to prefer only the largest and coldest of tundra streams in this region. Each of the 11 sample streams occupies one of three distinct alpine islands separated by lower-elevation areas. Each stream also occupies one of three major watersheds, defined by US Geological Survey cataloguing units having eight-digit hydrologic unit codes (http://water.usgs.gov/GIS/huc.html), including the Cache la Poudre, Big Thompson, and St Vrain basins, all tributaries of the South Platte River. Watersheds are not geographically correlated with alpine islands because high alpine areas often divide the headwater reaches of multiple drainage basins (Fig. 1a).

Five of the 11 sample streams occupy an alpine island in the vicinity of Hague's Peak (hereafter termed the Hague's region) in northern RMNP; four streams occupy an alpine island in southern RMNP in the area surrounding Long's Peak; and the final two streams are found on opposite sides of Niwot Ridge, *c*. 12 km south along the continental divide of the RMNP boundary (Fig. 1a). As of this study, these 11 streams harboured the only known populations of this species in the RMNP area. The group is located within a relatively small spatial extent of < 800 km^2.

We collected *P. neomacropyga* in early-mid August 2003, the time of year when larvae are large and nearing pupation. Larvae were collected from the bottoms of cobbles and boulders in areas of fast flow and immediately preserved in 75% ethanol. These were transferred back to the laboratory, taxonomically verified, and stored at –20 °C prior to DNA extraction.

Genetic typing

Total genomic DNA was isolated from 50 to 60 individuals per sample stream using a basic salt extraction method and ethanol precipitation (Black & DuTeau 1997). Pellets were resuspended for storage at –20 °C in 200 μL Tris-EDTA. Polymerase chain reaction (PCR) amplified a 307-bp fragment comprising the extreme 3′ end of the mitochondrial cytochrome oxidase subunit I (COI) gene. Fragments 100–400 bp in length are ideal for single-stranded conformation polymorphism (SSCP, Orita *et al*. 1989), the primary method we used to screen for sequence variation. Primers followed Lunt *et al*. (1996), including a version of their forward primer UEA9 modified for *P. neomacropyga* (5′-GTAAACATCACATTCTTCCCACAACA-3′), and their unmodified reverse primer UEA10, which includes 22 bp of the 5′ end of the adjacent tRNA-leucine.

Each PCR was run in a 50 μL total volume containing 43.8 μL ddH_2O, 5 μL 10× buffer (with 15 mM $MgCl_2$), 0.1 μL 20 mM dNTPs, 0.1 μL each of 500 μM primers, and 1 μL template DNA covered in autoclaved light mineral oil. This mixture was heated to 94 °C for 5 min, followed by addition of 0.2 μL *Taq* polymerase at 80 °C. PCR consisted of 35 cycles of 94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 6 min.

We used SSCP to assess sequence variation among PCR products following the general protocol outlined by Hiss *et al*. (1994) and Black & DuTeau (1997). Initially, we ran all products on a 38 × 50 cm nondenaturing polyacrylamide gel containing 5% acrylamide and 5% glycerol. In order to

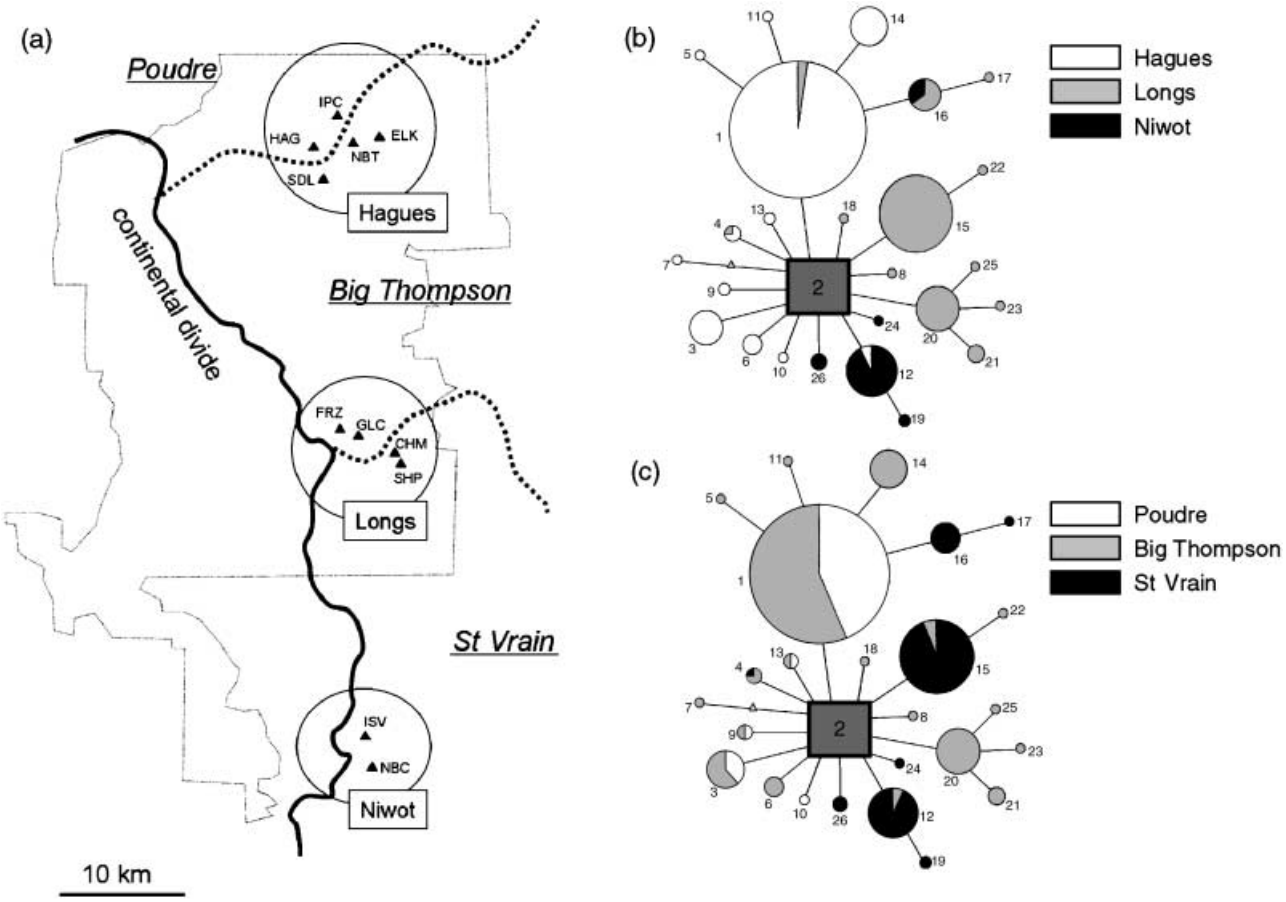

Fig. 1 (a) Map of study region. Thin broken line shows boundary of Rocky Mountain National Park, solid bold line is continental divide, broken bold lines are major watershed boundaries (watershed names are underlined), large circles approximate 'islands' of alpine tundra (labelled in boxes), and small triangles are sample streams with three-letter codes. (b,c) Haplotype network indicating distributions among alpine islands (b) and watersheds (c). Size of each circle is proportional to total haplotype abundance, and pie slices represent relative frequencies in each island/watershed *except* for haplotype 2, the ancestral haplotype, which was very abundant and found in significant proportion in all sample sites (see Table 1). Small triangle represents a hypothesized unsampled haplotype.

minimize the rate of false positives (different banding pattern but same sequence) and false negatives (same banding pattern but different sequence), we directly sequenced the purified PCR products (QIAquick PCR Purification Kit, QIAGEN) of at least three individuals showing each distinct banding pattern per population using an ABI 3100 Genetic Analyser. For less common banding patterns, all individuals were sequenced. Results from the 5%/5% gel revealed no false positives, but a few false negatives (*c.* 1 in 8 banding patterns comprised > 1 unique sequence). We therefore further ran all SSCPs on a gel containing 8.5% acrylamide and 4% glycerol and repeated the above sequencing procedures. Using the combination of 5%/5% and 8.5%/4% gel mixtures, no false positives or false negatives were revealed, and unsequenced individuals were assigned a sequence according to their combined SSCP banding patterns.

Analyses

All sequences were aligned manually using BIOEDIT (Hall 1999), and we used ARLEQUIN 2.000 (Schneider *et al*. 2000) for exploratory analyses of sequence variation. For each sample stream, genetic diversity was calculated in ARLEQUIN as the probability that two randomly chosen haplotypes are different (analogous to heterozygosity for diploid loci).

Spatial structure of genetic variation was further assessed using a nested analysis of molecular variance (AMOVA) in ARLEQUIN. Nesting was imposed in one of two ways. First, we tested the null hypothesis that population genetic structure was not associated with major watershed by grouping each local population according to its watershed location. Rejection of this hypothesis would provide evidence that major watershed boundaries have been barriers to gene flow. Second, we grouped populations according to

Table 1 Sample size (*N*), genetic diversity, and numbers of each haplotype found in each population. Shaded haplotypes are private

Alpine area:	Hague's					Long's				Niwot	
Site:	IPC	HAG	ELK	NBT	SDL	FRZ	GLC	CHM	SHP	ISV	NBC
N:	48	50	48	49	50	50	49	49	49	47	51
Diversity: haplotype	0.393	0.465	0.680	0.735	0.716	0.669	0.194	0.624	0.547	0.429	0.566
1	36	5	20	22	9		2				
2	11	36	15	9	23	14	44	11	25	35	23
3	1	6			11						
4					3			1			
5				1							
6				7							
7				1							
8						1					
9		1			1						
10		1									
11			1								
12					2					2	25
13		1			1						
14			12	9							
15						1	2	27	21		
16								9	2	6	
17								1			
18							1				
19											2
20						25					
21						6					
22						1					
23						1					
24											1
25						1					
26										4	

occupancy of alpine islands, in which case rejection of the null hypothesis would suggest that gene flow has occurred significantly more often within than between islands. Distance-based fixation indices (as per Weir & Cockerham 1984) were tested for significance using 100 000 permutations of the data.

For both of the nesting schemes, there was a potential problem of unequal number of populations per group. In particular, there were only two populations in the Poudre watershed, and similarly only two populations in the Niwot island group (see Fig. 1a); therefore, there was some concern that statistical power to detect significance was lacking. To help overcome this problem, we repeated both AMOVAs described above using only the two largest groups in their respective category (watershed or island). Given true among-group population structure, fixation indices are expected to increase to significant levels after excluding the smallest group.

Because COI is a coding gene, it is subject to natural selection and may violate the neutrality assumption necessary for phylogeographic inference (Ballard & Whitlock 2004). We therefore tested the null hypothesis of neutrality using Tajima's (1989) *D* statistics implemented by the software DNASP 4.10.4 (Rozas *et al*. 2003). An initial test included all individuals sampled across the study area. Panmixia, however, is a key assumption of Tajima's test. Due to strong biological implication that this group is not panmictic (see 'Study organism' subsection), we further implemented neutrality tests on each of the 11 populations individually in addition to more inclusive groups of geographically proximate populations as defined by location on an alpine island. Large sample sizes (Table 1) allowed robust tests at the finer scales.

We constructed a haplotype network using the software TCS 1.21 (Clement *et al*. 2000) based on the statistical parsimony method described by Templeton *et al*. (1992). Reticulations were resolved following common theoretical predictions about network structure (Crandall & Templeton 1993; Posada & Crandall 2001). In order to visualize differences between geographically grouping streams

according to watershed vs. alpine island, the network was coded in two ways, each depending on the geographical structure of interest (see Fig. 1b, c).

We implemented a nested clade analysis (NCA, Templeton *et al*. 1992) using the most recently developed inference key (Templeton 2004) after statistically evaluating the relationship between the TCS-generated network and geography using GEODIS software (Posada *et al*. 2000). Although there are drawbacks to NCA that include inability to assign statistical confidence to qualitative inferences from the key (e.g. see Knowles & Maddison 2002), we used it here in an attempt to discriminate broadly between more recent events (e.g. population expansion or ongoing gene flow) vs. historical processes (e.g. allopatric fragmentation) as drivers of current phylogeographic structure.

Further, we looked for evidence of IBD following Rousset (1997) using matrices of pairwise linearized F_{ST} [$F_{ST}/(1-F_{ST})$, Slatkin 1995] and natural logarithm of spatial distances in a Mantel test using Bohonak's (2002) IBD program. Initially, we used straight-line (Euclidean) distance to compare IBD patterns between two spatial scales, the broader including all pairs of sample sites (55) and the finer including only pairs in the most site-rich alpine island (Hague's region, 10 pairs). We included both scales because strong dispersal barriers can cause a breakdown of IBD patterns due to the combined effect of random genetic drift and lack of migration (Slatkin 1993); however, if dispersal is indeed extremely limited, IBD ought to be evident across a smaller spatial extent (e.g. Keyghobadi *et al*. 2005).

In addition to testing for genetic isolation by Euclidean distance, we also tested for IBD among populations in the Hague's region using three other biologically realistic distance measures. We calculated these distances by estimating pairwise least-cost pathways between populations according to spatial models incorporating landscape elements important to aquatic insect flight dispersal. Number of landscape parameters increased additively in each successive model as listed in Table 2. A 10×10 m digital elevation model (DEM) was the base layer for these models, and we used ARCGIS (ESRI 2005) to assign costs to each cell based on predicted relative importance of associated landscape elements to insect flight. Resulting pairwise least-cost distance values and genetic distances were then used in a Mantel test of IBD as described above, and we compared fit and significance of the relationship for each competing model.

In the most complex model (#4, Table 2), per-cell costs of elevation difference and slope were linearly normalized 0–1, and cells on ridge-tops were assigned an additional two cost units; for each cell, the sum of all costs gave its total cost (see Fig. 2). The ridge-top cost was doubled for two reasons: (i) slope cost decreases substantially on ridge tops (making these apparently more preferred locations for movement), but at the same time, (ii) ridge-tops were assumed to have higher wind speed than any other location on the grid (see Liston & Sturm 1998). Model #4 included all key factors thought to be influential to insect flight in the alpine zone; however, each individual factor was given a normalized and linear effect on flight cost in order to minimize complex and potentially unrealistic assumptions.

Results

We identified a total of 26 haplotypes among the 11 populations, with sample size 47–51 individuals per population for a total of 539 individuals analysed (Table 1). All sequences are deposited in GenBank under Accession nos DQ334672–DQ334697. Twenty-three of the 307 nucleotide sites were variable due to substitutions; of these, there were 20 transitions and three transversions, and six were nonsynonymous substitutions. As is common for insect mtDNA, nucleotide frequencies were AT-biased (freq. T = 0.34, C = 0.23, A = 0.29, G = 0.14). No significant deviations from neutrality were revealed among sequences at the population level (range $D = -1.46$ to $D = 0.58$; all $P > 0.10$), the alpine island level (range $D = -1.21$ to $D = -0.72$; all $P > 0.10$) or across the entire study area ($D = -1.66$; $0.10 > P > 0.05$).

Population structure

Genetic diversity values ranged from 0.194 (GLC, Long's region) to 0.735 (NBT, Hague's region) across the 11 sample streams (Table 1). Average diversity varied but was not significantly different either between alpine islands (0.60, 0.51, 0.50 for Hague's, Long's, and Niwot, respectively) or between major watersheds (0.43, 0.60, 0.54 for Poudre, Big Thompson, and St Vrain, respectively). Private haplotypes (those found in a single location) occurred in eight of the 11 sample populations; indeed, 16 of the 26 total haplotypes identified were private (Table 1). Additionally, all pairwise F_{ST} values were significant ($\alpha = 0.05$) except two (NBT-ELK and HAG-SDL, see Appendix), and these were pairs of populations that were geographically proximate without major topographical barriers.

Across the 11 populations, AMOVA revealed that grouping streams by major watershed did not explain a significant proportion of genetic variation ($F_{CT} = 0.014$, $P = 0.33$, Table 3a). A reduced AMOVA excluding the two-population Poudre watershed revealed a similar pattern ($F_{CT} = 0.05$, $P = 0.16$). Grouping streams by alpine island, however, explained a significant 13.6% ($P = 0.01$) of total variation (Table 3b). Exclusion of the smallest group (Niwot) from this analysis further increased the magnitude of this effect ($F_{CT} = 0.16$, $P = 0.03$), implying that dispersal has been more limited across extensive low-elevation forested areas than across high-elevation watershed boundaries. In the significant full model, a further 24.6% ($P < 0.0001$) of

Table 2 List of model parameters and assumptions of four models used to estimate pairwise distances between sites, with reasoning behind each and supporting references. Models 1–3 yield minimum distance under respective assumptions; see text for further explanation of Model 4

Model #	Assumptions/ costs of landscape features	Reasoning	References
1	Euclidean distance (straight line on map)	Simplicity	most prior IBD studies
2	Minimum surface distance	Flight does not occur at great distances above the earth	Adler *et al*. (1983) Choe *et al*. (1984)
3	Travel along stream = 0 cost + minimum surface distance	Flight of stream insects is concentrated along the stream, rather than inland	Thompson (1976) Flecker & Allan (1988) Petersen *et al*. (1999, 2004) Briers *et al*. (2002) Macneale *et al*. (2005)
4	Travel along stream = 0 cost	Flight of stream insects is concentrated along the stream, rather than inland	Thompson (1976) Flecker & Allan (1988) Petersen *et al*. (1999, 2004) Briers *et al*. (2002) Macneale *et al*. (2005)
	Difference in elevation from stream increases cost; related to:		
	Air temperature	Flight activity is positively correlated to air temperature, ceasing in many cases below *c*. 7 °C	Shipp *et al*. (1988) Waringer (1991) Kuusela & Huusko (1996) Briers *et al*. (2003)
	Wind speed	Flight activity is negatively correlated with wind speed	Roberts & Irving-Bell (1996) Briers *et al*. (2003)
	Steeper slope of terrain increases cost; related to:		
	Vegetation density	Flight activity is positively correlated with vegetation density, especially in dry climates where vegetation may increase local relative humidity	Jackson & Fisher (1986) Myers *et al*. (2001) Briers & Gee (2004)
	Ridge tops = double cost	Dramatic increase in wind	Liston & Sturm (1998) Roberts & Irving-Bell (1996) Briers *et al*. (2003)

genetic variation was distributed among streams within alpine areas, and the remaining 61.8% was due to within-stream variation. Overall F_{ST} was significant ($P < 0.0001$) and relatively large at 0.38 (Table 3b).

The haplotype network was relatively shallow with a maximum of five mutational steps between haplotypes (Fig. 1b, c). Haplotype 2 had strong support as the ancestral haplotype due to its representation in a significant proportion of individuals in all populations (see Table 1) and its central location in the network. Only a single unsampled haplotype was suggested, this linking haplotype 2 to a singleton (Fig. 1b, c). We have presented the same tree in two ways: first, by representing haplotype distribution proportionally according to alpine island (Fig. 1b), and second, according to major watershed (Fig. 1c). Of the 26 total haplotypes, 16 were private and therefore fully represented in a single alpine island or watershed. Conversely, the presumed ancestral haplotype was found in all populations and therefore was present in all alpine islands and watersheds. Of the nine remaining haplotypes, seven were distributed across > 1 watershed (Fig. 1c) and only four across > 1 alpine island (Fig. 1b). Furthermore, the second most common haplotype (#1) was dominant in a single alpine island (Hague's) but was shared nearly equally between two watersheds (Poudre and Big Thompson).

For NCA, the shallow nature of the network allowed only 1-step and 2-step clades to be delineated (Fig. 3), and there were only two 2-step clades. Still, several of the clades revealed significant geographical association (Fig. 3, Table 4). As a general pattern, more recent events (e.g. range expansion and restricted gene flow with IBD) were inferred for smaller and less spatially extensive clades, and historical processes (e.g. allopatric fragmentation and/or past gene flow followed by extinction of intermediate populations) for more inclusive and geographically extensive clades (Table 4). For the total cladogram, results were inconclusive, due in large part to the shallow network and lack of interior clades at this level.

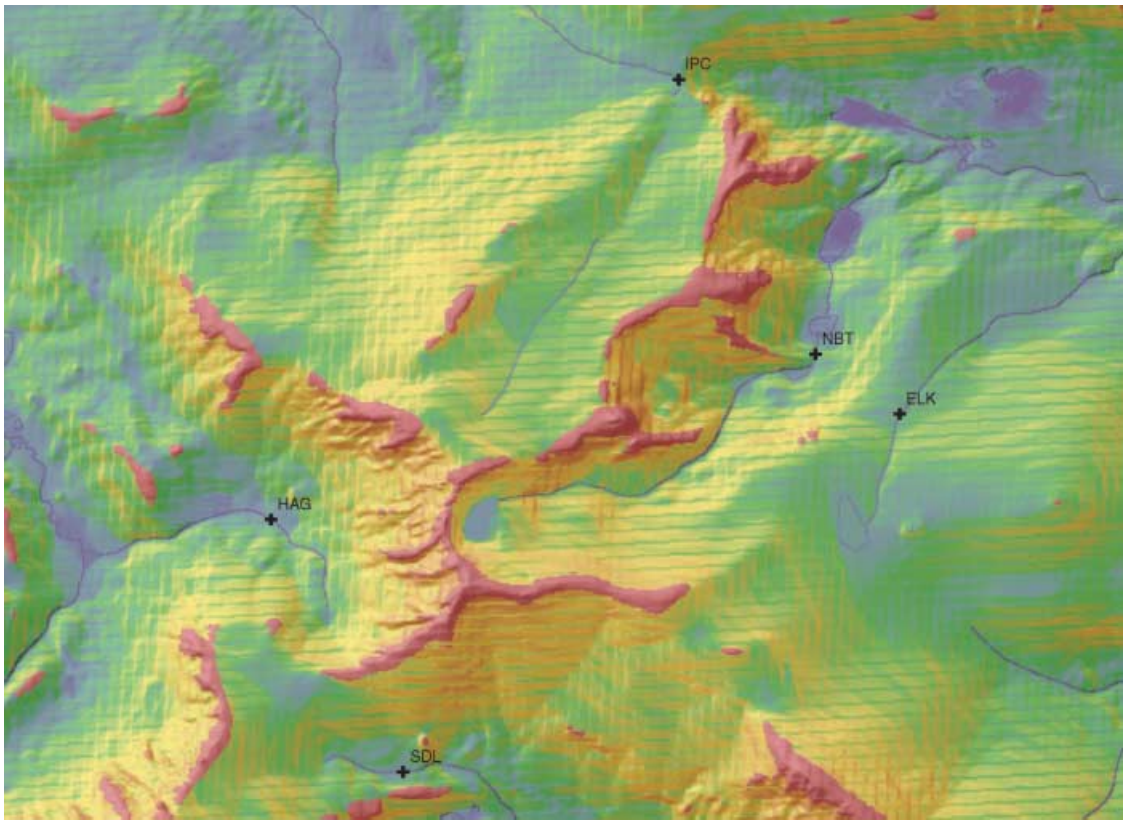

Fig. 2 Map of the Hague's region alpine island showing the cost surface according to Model 4 (see Table 2). Cost increases along the colour spectrum from blue (least cost) to red (greatest cost). Sample streams are marked with + and three-letter code.

Table 3 AMOVA variance components, percentage variation explained at each hierarchical spatial level, and fixation indices for (a) grouping populations according to major watershed occupied, and (b) grouping according to alpine island

(a)

Source of variation	d.f.	Variance components	Percentage variation	Fixation indices
Among watersheds	2	0.008 (Va)	1.36	F_{CT} [Va/Vt] = 0.014 (P = 0.33 NS)
Among streams within watersheds	8	0.20 (Vb)	34.41	F_{SC} [Vb/(Vb + Vc)] = 0.35 (P < 0.0001)
Within streams	528	0.38 (Vc)	64.23	
Total	538	0.58 (Vt)		F_{ST} [(1 – Vc)/Vt] = 0.36 (P < 0.0001)

(b)

Source of variation	d.f.	Variance components	Percentage variation	Fixation indices
Among alpine islands	2	0.083 (Va)	13.64	F_{CT} [Va/Vt] = 0.14 (P = 0.010)
Among streams within islands	8	0.15 (Vb)	24.58	F_{SC} [Vb/(Vb + Vc)] = 0.28 (P < 0.0001)
Within streams	528	0.38 (Vc)	61.78	
Total	538	0.61 (Vt)		F_{ST} [(1 – Vc)/Vt] = 0.38 (P < 0.0001)

Isolation by distance

Using Euclidean distance, IBD was evident at both spatial scales analysed (Fig. 4). At the broad scale (across all pairs of sites), the relationship was weak (r = 0.34) but significant (P = 0.006, Fig. 4a). At the finer scale including only collections in the Hague's region, the relationship was strong (r = 0.80; P = 0.04) despite significantly smaller sample size (Fig. 4b). Indeed, pairs of sites occupying the same alpine island may be driving positive IBD at the broad scale, as evidenced by comparing the within- vs. among-island sections of the broad-scale plot (Fig. 4a). There is a positive

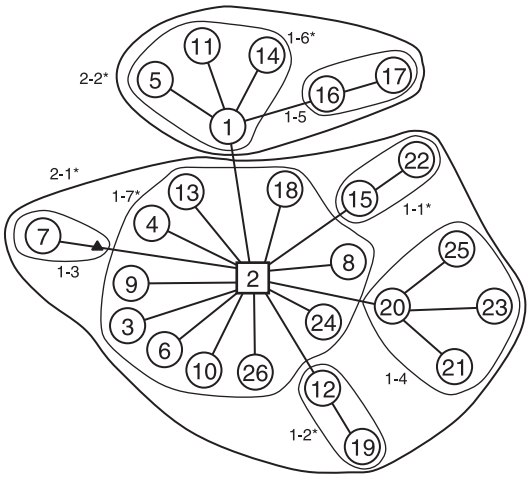

Fig. 3 Simplified haplotype network from Fig. 1 with clades delineated for nested clade analysis. Asterisks mark clades with significant geographical associations.

relationship among sites sharing an alpine island but no relationship for pairs occupying different islands.

Effective pairwise distances estimated by each of the four models of landscape connectivity produced similar IBD results (Table 5). Euclidean distance showed stronger correlation to genetic distance than did any of the distances determined by the putatively more biologically realistic connectivity models. Estimated effective distances were highly correlated between all models (Table 5), however, and each model produced significant ($\alpha = 0.05$) Mantel results with r having a narrow range among them ($r = 0.71$ for Model 2 to $r = 0.80$ for Model 1).

Discussion

Our results demonstrate a high degree of genetic structure for *Prosimulium neomacropyga* among 11 alpine streams in the Rocky Mountain National Park area, Colorado. Across the study region, this structure is significantly associated with high-elevation 'islands' of alpine tundra, suggesting that dispersal between populations is limited more by extensive lower-elevation areas than by major drainage divides for this strictly alpine species. These results are similar to those from studies of terrestrial alpine insects in the Rocky Mountains (e.g. Knowles 2001; DeChaine & Martin 2004) and as such may reflect the isolation of patches of alpine habitat during climatic warming following the most recent glacial recession (see Elias 1996).

Although our study has limited ability to infer the specific processes that have contributed to current genetic structure, the NCA lends some support to a historical fragmentation hypothesis. In general, the shallowness of the haplotype network led to less conclusive NCA results for progressively higher clade levels; however, the most conclusive result for a 2-step or higher clade was allopatric fragmentation for clade 2-2 (Table 4). Further, the other 2-step clade and the geographically widespread 1-step clade (1-7) both suggested as one possibility past gene flow followed by extinction of geographically intermediate populations, a situation that could result from historical habitat fragmentation. Conversely, recent and ongoing events (range expansion and restricted gene flow/IBD) were inferred for the other significant 1-step clades (1-1 and 1-6) that were predominantly confined to a single alpine island. These results suggest that dispersal events, though infrequent, continue to be an important influence on population genetic structure at the within-island but not between-island scale.

Across the whole study area, however, there was an indication of IBD, a pattern that could be interpreted as limited ongoing dispersal at the between-island scale. Typically, if strong dispersal barriers exist, IBD is not detected (e.g. Slatkin 1993; Keyghobadi *et al*. 2005). Given the weak vs. strong relationships at the respective broad vs. fine spatial scales (Fig. 4), however, the broad-scale IBD pattern is likely driven by the stronger relationship of genetic to geographical distance at the finer, within-island scale. Furthermore, broad-scale IBD may be an imprint of historical gene flow (see Garnier *et al*. 2004) if alpine populations were geographically more widespread in a cooler Pleistocene climate.

Table 4 Inferences of nested clade analysis. Only clades with significant geographical relationships are included (see Fig. 3)

Nested clade	Inference key steps	Conclusion
1-1	1, 2, 11, 12, No	Contiguous range expansion
1-6	1, 2, 3, 4, No	Rstricted gene flow, IBD
1-7	1, 2, 3, 5, 6, 7, 8, Yes	Restricted gene flow but with some long-distance dispersal over intermediate areas; OR past gene flow followed by extinction of intermediate populations
2-1	1, 2, 3, 5, 6, 7, 8, Yes	Restricted gene flow but with some long-distance dispersal over intermediate areas; OR past gene flow followed by extinction of intermediate populations
2-2	1, 19, No	Allopatric fragmentation
Total	1, 2, ?	No interior clades; inconclusive outcome

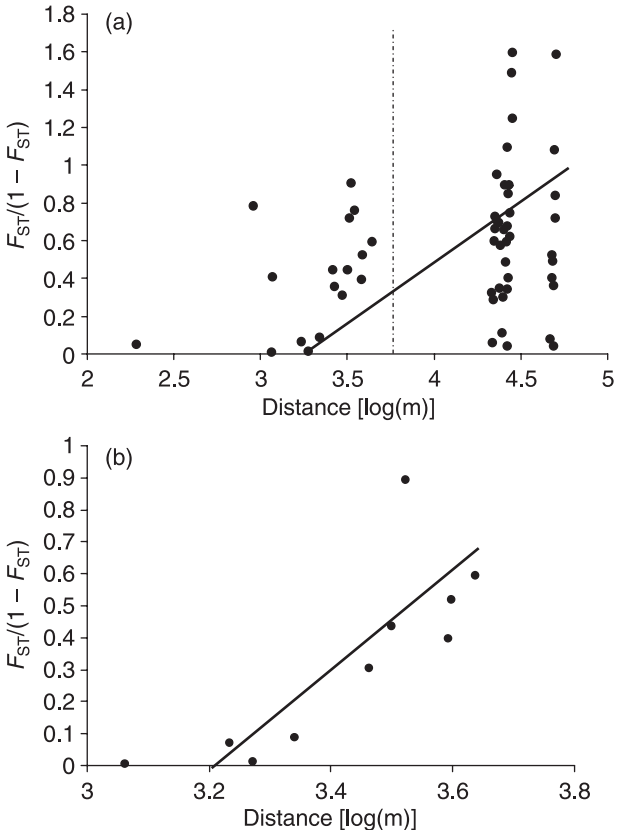

Fig. 4 Isolation by [Euclidean] distance evident at two spatial scales. Each point represents one unique pair of populations; trendlines according to reduced major axis regression in IBD program (Bohonak 2002). (a) All populations included (55 pairs); vertical broken line indicates spatial break between pairs occupying same (left of line) vs. different alpine islands. (b) Only populations within the Hague's alpine island included (10 pairs). See Table 5 for Mantel statistics.

At the finer spatial scale, *P. neomacropyga* populations within the single most populated alpine island (Hague's region) indeed demonstrated strong IBD (Fig. 4b, Table 5). The only two pairs of populations (NBT-ELK and HAG-SDL) that did not have a significant pairwise F_{ST} occupied proximate streams all within the Hague's region (Figs 1 and 2). As with the AMOVA and NCA inferences, these results also suggest limited dispersal occurring within but not among islands.

Given the evidence of fine-scale genetic isolation by Euclidean distance, we anticipated that distances generated by our presumably more biologically realistic spatial models would improve the fit of the IBD relationship. Of the four models compared, Euclidean distance surprisingly provided the best fit, suggesting that geographical proximity of locations is the most important determinant of gene flow regardless of presence/absence of apparent dispersal barriers. Importantly, however, pairwise distances obtained by each of the other models were strongly correlated with Euclidean distance. As such, we cannot disentangle the influences of straight-line distance vs. more realistic barriers, but we can suggest that Euclidean distance is a reliable surrogate for other connectivity measures that include environmental features with demonstrated effects on insect flight (see References in Table 2). The correlation between distance measures is likely system- and scale-specific, however, and cannot be expected to hold in all cases. In relatively small areas of steep mountainous terrain, it makes sense that sites that are more geographically distant are also more likely separated by landscape features that may inhibit flight dispersal, such as high ridgelines and more extensive areas lacking stream and riparian dispersal corridors (see, e.g. Fig. 2). As spatial extent increases, however, coarse-grain heterogeneity is also likely to increase (Wiens 1989) and the positive relationship between Euclidean distance and more biologically realistic dispersal routes is less likely to hold.

In general, our results are comparable to several other studies of stream insect population genetic structure that have suggested some movement among streams within a reasonably small spatial extent, often accompanied by a break in the pattern associated with various types of stronger dispersal barrier encountered as spatial extent increases (Smith & Collier 2001; Wilcock *et al*. 2001, 2003; Monaghan *et al*. 2002; Schultheis *et al*. 2002; Wishart & Hughes 2003). These are in contrast to studies showing the other common pattern identified in stream insects: a strong differentiation of populations within individual streams

	Mantel			
IBD model	*N*	*r*	*P*	
Broad scale (Euclidean)	55	0.34	0.004	
Fine scale				
Model 1 (Euclidean)	10	0.80	0.036	
Model 2 (surface dist.)	10	0.71	0.018	$r = 0.933$
Model 3 (streams + surface)	10	0.72	0.025	$r = 0.942$
Model 4 (full model)	10	0.72	0.024	$r = 0.926$

Table 5 Summary of Mantel correlations (Pearson's r) and significance for the broad scale (all populations, Euclidean distance only) and the four alternate connectivity models at the fine scale (Hague's region only). Last column: correlations of respective model to Model 1 (Euclidean)

resulting in lack of significant spatial structure among streams (Schmidt *et al*. 1995; Bunn & Hughes 1997; Hughes *et al*. 1998, 2000, 2003a; Monaghan *et al*. 2002). This pattern has been attributed to different sample 'populations' along a stream corridor being the results of only a few matings ('patchy recruitment', Bunn & Hughes 1997), combined with minor larval dispersal barriers along the stream. This pattern is unlikely to occur (and impossible to test) for *P. neomacropyga* alpine populations because of its extremely limited longitudinal distribution (only a single spatially continuous population was identified on each sample stream). Furthermore, the more temporally synchronous emergence of this (and most other) species in the US Rocky Mountains makes the kind of nonrandom mating necessary for patchy recruitment unlikely (cf. Hughes *et al*. 2003b).

Compared to other studies of stream insect mtDNA population genetic patterns across spatial scales broad enough to include > 1 major watershed, our study of *P. neomacropyga* suggests an unusually strong pattern of genetic subdivision (overall F_{ST} = 0.38). Typically, F_{ST} values have fallen in the range from insignificant (e.g. for a widely dispersing caddisfly in southeastern Australia, Baker *et al*. 2003) to significant but low values ranging from *c*. 0.08–0.15 (baetid mayflies in both southeastern Queensland and the US Rocky Mountains, Hughes *et al*. 2003a, 2003b; and a stonefly in the Appalachians, Schultheis *et al*. 2002). One exception has been a highly local-habitat-specific South African blepharicerid, in which overall F_{ST} was 0.94 and time since divergence was estimated at 2–3.5 million years (Wishart & Hughes 2003) between groups of populations separated by < 100 km. Aside from this notable exception, however, *P. neomacropyga* occupying alpine islands distributed across a relatively small spatial extent has one of the most significant levels of population structure yet recorded for a stream insect. Both strong dispersal barriers and habitat specificity to the alpine reaches of streams probably contribute to the lack of gene flow. Additionally, flight activity is probably limited in this species relative to other black flies due to its autogenous nature.

Isolation among high-alpine stream reaches likely has had similar effects on other alpine stream insects with limited dispersal potential. Confronted with potential broad-scale anthropogenic disturbances such as rapid climate change and atmospheric deposition in high-elevation Southern Rockies ecosystems (e.g. Baron *et al*. 2000; Welker *et al*. 2001), such species may have little capacity to respond and may be faced with local extinction. Oddly, despite the importance of headwater streams to whole-watershed biodiversity and ecosystem function (cf. Lowe & Likens 2005), there have been relatively few studies focused on the dispersal potential and genetic diversity of headwater species. The results of our study point to the need for further research on alpine stream species distributions, on their potential for evolutionary response to change, and on methods to understand dispersal between isolated habitat patches.

Acknowledgements

Special thanks to Peter H. Adler for his extensive knowledge of and enthusiasm for the Simuliidae, and for providing taxonomic verification of *Prosimulium neomacropyga* larvae. Thanks also to selfless folks in the Black lab who helped with molecular methods, especially Karen Fleming, Norma Gorrochotegui-Escalante, and Kristine Bennett. The CU Mountain Research Station (especially Bill Bowman) helped provide access to the N. Boulder Creek headwaters, and thanks to Rocky Mountain National Park administrators for collection permits there. Dave Pepin, Christine Albano, Julia McCarthy, Tom Hobbs, John Wiens and anonymous referees provided valuable comments on earlier versions of the manuscript. D. Finn was funded by an EPA-STAR graduate fellowship and a small grant from the Colorado Mountain Club Foundation.

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Deb Finn is interested in controls on the spatial distribution of biotic diversity in headwater streams, both at the population genetic and the community levels. This work is a part of her dissertation research at Colorado State University. David Theobald is a research scientist at the Natural Resource Ecology Lab and associate professor in the Department of Natural Resources Recreation and Tourism at CSU. He studies landscape change and its effect on the conservation of biodiversity. Bill Black is an insect geneticist who works on the population biology of high altitude snowpool mosquitoes. N. LeRoy Poff is an associate professor who studies the how the structure and functional composition of stream and river communities varies along environmental gradients. LeRoy was Deb's major graduate adviser.

Appendix

F_{ST} for all pairwise population comparisons. All values were significant at $\alpha = 0.05$ except those in bold

	ELK	HAG	IPC	SDL	NBT	CHM	SHP	FRZ	GLC	NBC
ELK										
HAG	0.3742									
IPC	0.0769	0.4729								
SDL	0.2861	**0.0101**	0.3445							
NBT	**0.0044**	0.3042	0.0657	0.2348						
CHM	0.3697	0.2863	0.4262	0.2573	0.3266					
SHP	0.4632	0.2532	0.5568	0.2391	0.3999	0.0460				
FRZ	0.5239	0.3943	0.5994	0.3631	0.4716	0.4188	0.4312			
GLC	0.4724	0.0369	0.6157	0.1014	0.3836	0.3012	0.2588	0.4421		
NBC	0.5216	0.3419	0.6134	0.2877	0.4595	0.4118	0.4194	0.4858	0.4001	
ISV	0.3305	0.0461	0.4170	0.0709	0.2688	0.2364	0.2279	0.3729	0.0546	0.2875