

Population genetic structure of a rare high-elevation black fly, *Metacnephia coloradensis*, occupying Colorado lake outlet streams

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SUMMARY

1. Using a portion of the mitochondrial cytochrome oxidase I gene, we evaluated the population genetic structure of a geographically rare black fly (*Metacnephia coloradensis*) that is a habitat specialist in outlet streams of large, productive, alpine lakes in Colorado, U.S.A. Given its rarity and life history traits that restrict dispersal, we hypothesised that genetic structure would show a signature of allopatric fragmentation associated with climatic warming since Pleistocene glaciations.
2. We tested for genetic isolation by distance (IBD) and applied nested clade analysis (NCA) to ask whether current genetic structure is primarily a consequence of historic fragmentation or if there is evidence of ongoing gene flow.
3. Only four populations were located despite a thorough search of potential sites, and they demonstrated a significant degree of genetic structure ($F_{ST} = 0.17$). However, there was some evidence of IBD in a plot of genetic versus geographic distance, and NCA further supported IBD and restricted ongoing gene flow in clades at all nested levels. Compared with a more widespread alpine black fly (*Prosimulium neomacropyga*) in the same region, *M. coloradensis* demonstrated significantly less population genetic structure.
4. Although these results counterintuitively implicate limited ongoing gene flow driving current population structure, significant IBD may be a signature of historic gene flow, especially if migration–drift equilibrium has not yet been reached since a late-Pleistocene fragmentation event. Extraordinarily dense local populations probably allowed *M. coloradensis* to maintain large effective population sizes and minimise genetic drift rates.
5. Despite large local populations, *M. coloradensis* is vulnerable to continued rapid environmental change because of its limited geographic distribution and high habitat specificity.

Keywords: allopatric fragmentation, alpine streams, gene flow, phylogeography, Simuliidae

Introduction

Many species that are currently confined to high-elevation ‘islands’ of alpine tundra habitat are believed to have been pushed to these extremes after the most recent glacial recession and associated

climatic warming (Elias, 1996; Hewitt, 2000), and much evidence suggests that climatic shifts during the Quaternary drove the contraction and expansion of populations along altitudinal gradients (e.g. Knowles, 2001; DeChaine & Martin, 2004). It is likely that alpine-specialist stream invertebrates have followed similar trajectories (e.g. Finn *et al.*, 2006). Today, the planet is in the midst of a warm interglacial period and probably has not experienced average temperatures this warm in more than 100 000 years (e.g.

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Williams *et al.*, 1998). Species presently restricted to small mountaintop islands likely were more widespread in the cooler past. Decreased gene flow associated with this presumed allopatric fragmentation is expected to lead to an increase in the spatial structure of populations, especially in species with low dispersal ability, and at its extreme could lead to speciation (e.g. Hewitt, 2000).

Population genetic studies can elucidate the degree of population subdivision among isolated habitats such as those occupying alpine islands. However, it can be difficult to tease apart the relative importance of past allopatric fragmentation versus current isolation by distance (IBD) in driving present-day genetic patterns (e.g. Garnier *et al.*, 2004; Smith & Farrell, 2005). For example, in a recent study of an alpine black fly (Simuliidae: *Prosimulium neomacropyga* Peterson) from Colorado, U.S.A., a high degree of population structure was suggested among alpine islands (Finn *et al.*, 2006). The conclusion was that the extensive low-elevation 'sea' surrounding the alpine islands acts as a strong dispersal barrier, and allopatric fragmentation was the primary driver of spatial genetic structure. However, IBD was also detected, a pattern that typically implicates a low level of ongoing gene flow among populations.

Occupying the same region in Colorado, *Metacnephia coloradensis* Peterson & Kondratieff is another alpine black fly that is geographically rarer, known only from the outlet streams of some large, productive tundra lakes (Adler, Currie & Wood, 2004). This high degree of habitat specialisation likely minimises its dispersal potential (cf. Wishart & Hughes, 2001, 2003). Additionally, at an ecologically relevant time scale, the lake outlets provide stable habitat, which is probably a key determinant of traits conferring reduced dispersal in insects (see Roff, 1990).

Metacnephia coloradensis has an additional suite of life-history traits that make long-distance dispersal unlikely. It mates on the ground immediately after emergence, is incapable of blood feeding (and therefore is obligately autogenous), and makes only infrequent short flights (Peterson & Kondratieff, 1995). These traits also characterise some aquatic insects in the environmentally similar Arctic tundra and are presumably adaptations to avoid risky dispersal into the harsh terrestrial environment (Downes, 1965). Given this combination of traits, *M. coloradensis* is among the least likely of stream

insects in this region to undergo long-distance dispersal. Hence, this species may provide a useful population genetic model for investigating patterns of postglacial allopatric fragmentation of alpine stream populations in the southern Rocky Mountains.

Prior to this study, *M. coloradensis* was known from only two locations, both in Colorado: the type locality (a lake outlet 3558 m a.s.l. in the North Boulder Creek drainage south of Rocky Mountain National Park in Boulder County) and an unspecified site near Mosquito Pass in Park County (based on a single pupa, Adler *et al.*, 2004). Although it is rare because of a limited geographic range in addition to narrow habitat tolerance (see Rabinowitz, 1981), population density at the type locality is spectacularly high (350 000 late larval instars m⁻², Adler *et al.*, 2004), and it has been reported as the most abundant arthropod in the drainage (Bushnell, Foster & Wahle, 1987). In late summer, larvae at this site coat all substrates so that the streambed appears itself to be an undulating organism. We refer to this extreme population density as the 'Medusa effect' (Fig. 1) because of the writhing, snakelike movements of the large larvae (final-instar length: 7.7–10.0 mm)



Fig. 1 *Metacnephia coloradensis* larvae approximately 1 month prior to emergence covering a protruding rock at the outlet of Green Lakes no. 5, North Boulder Creek. Photo by Jeremy Monroe.

covering stones removed from the stream. Because of its abundance at the type locality, we believed that *M. coloradensis* would be found in other characteristic habitats in the region, and our initial efforts in this study included a thorough survey of such localities (outlets of large, high-elevation lakes).

The primary objective of the current study was to assess the degree of genetic structure among all extant populations of *M. coloradensis*, based on sequence variation within the mitochondrial cytochrome oxidase subunit I (COI) gene. Additionally, we used two complementary methods to assess the weight of evidence for allopatric fragmentation versus ongoing gene flow in contributing to observed population genetic patterns. Finally, we compared aspects of population structure between *M. coloradensis* and *P. neomacropyga*, which is also confined to alpine streams in Colorado but is not limited to lake outlets. We expected a higher degree of population structure for *M. coloradensis* than for *P. neomacropyga* because of its greater geographic rarity, habitat specialisation, and presumed lower dispersal capabilities. Given these characteristics, we also hypothesised that the population structure of *M. coloradensis* would be driven primarily by postglacial allopatric fragmentation and

subsequent genetic drift in local populations, and that evidence for ongoing gene flow would be minimal.

Methods

Study area and data collection

We focused collection efforts for *M. coloradensis* at lake outlets in alpine areas patchily distributed to the north and south of the species' type locality (North Boulder Creek, Fig. 2). Collections were made from early August to early September 2003 and included a collection from North Boulder Creek. Simuliid larvae were collected from cobbles, boulders, and occasional bedrock at each site. They were fixed in 75% ethanol in the field, sorted to species in the lab, and stored in fresh ethanol at -20°C prior to DNA extraction. Voucher specimens are deposited in the Clemson University Arthropod Collection and the C.P. Gillette Museum of Arthropod Biodiversity at Colorado State University.

Genetic typing

Total genomic DNA was isolated from individuals of *M. coloradensis* using a basic salt extraction method

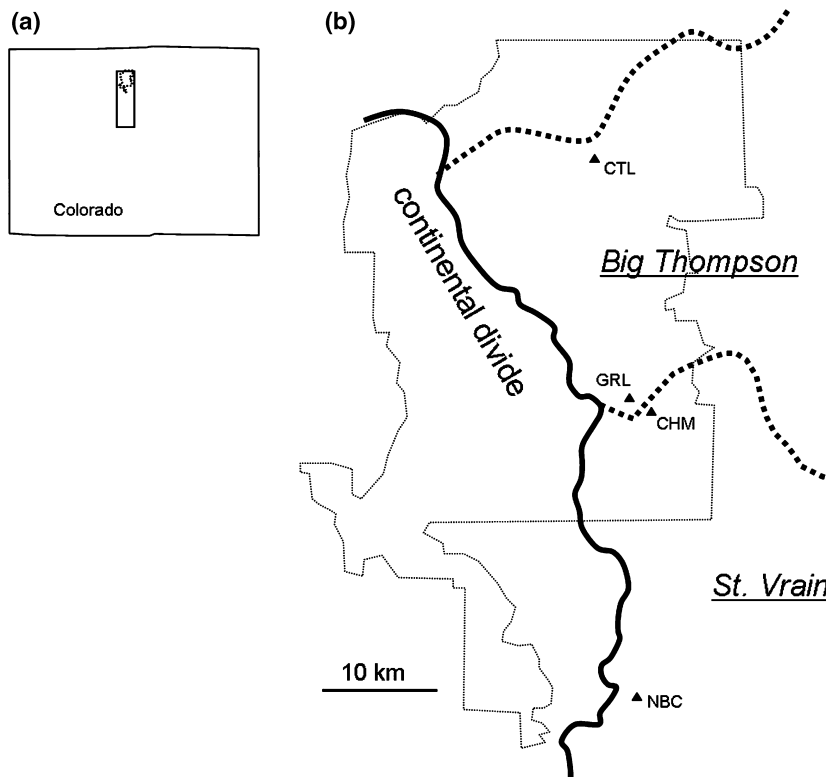


Fig. 2 (a) State of Colorado (U.S.A.), illustrating approximate extent of sampling (solid rectangle) for *Metacnephia coloradensis* along the north-south oriented cordillera. All sample sites are listed in Table 1. Dashed line represents Rocky Mountain National Park (RMNP) boundary. (b) Magnified view of RMNP area (thin dashed line), with locations of *M. coloradensis* populations (triangles) and major watershed boundaries (thick dashed lines). Watershed names are underlined. The divide between sites GRL and CHM is a ridgeline that reaches >4200 m a.s.l.

followed by ethanol precipitation (Black & DuTeau, 1997). We used primers UEA9 and UEA10 (Lunt *et al.*, 1996) to PCR-amplify a fragment of the mitochondrial COI gene comprising 307 bp (primer sequences included) at the rapidly evolving extreme 3' end of the gene. PCR protocols followed those outlined by Finn *et al.* (2006) and included an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 51 °C for 1 min, and 72 °C for 1 min, with a final extension step of 72 °C for 6 min.

We used single-stranded conformation polymorphisms (SSCP, Orita *et al.*, 1989) to assess sequence variation among PCR products, following the specific procedures outlined by Hiss *et al.* (1994) and Black & DuTeau (1997). The SSCP method is a simple, inexpensive, and sensitive way to assess sequence differences among a large number of individuals that therefore facilitates analysis of the large sample sizes often essential for population genetics analysis (cf. Hiss *et al.*, 1994; Sunnucks *et al.*, 2000). The procedure is based on the principles that a denatured (single-stranded) DNA molecule will conform into one or more stable shapes because of secondary base-pairing, that variation in sequence produces variation in these shapes, and that mobility of a molecule in a non-denaturing gel is determined by both its size and shape. The sensitivity of SSCP is due to the sensitivity of the 3-D shapes to even single-point mutations in a DNA sequence. Despite these sensitivities, false positives (different banding but same sequence) and false negatives (same banding but different sequence) can occur (Chakravarty, Redkar & Mittra, 1996) but can be avoided by using a controlled analysis strategy.

We ran replicates of all SSCP products on each of three non-denaturing polyacrylamide gels containing different acrylamide/glycerol concentrations. Initially, we ran all samples on a 5% acrylamide/5% glycerol gel prepared as described by Hiss *et al.* (1994). We then used an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.) to sequence directly at least three individuals per sample site showing each distinct banding pattern. (For banding patterns less common than three individuals per site, all individuals were sequenced.) Because of some false negatives (within approximately one in eight visually different banding patterns) on the 5%/5% gel mixture, all individuals were further run on both 7.5% acrylamide/5% glycerol and 8.5% acrylamide/4% glycerol gels. Using this combination of

gel mixtures, we could resolve all sequence variation, and unsequenced individuals were assigned a sequence according to their combined SSCP banding patterns. No false positives were identified.

Analyses

All sequences were aligned manually using BioEdit (Hall, 1999), and we used Arlequin 2.000 (Schneider, Roessli & Excoffier, 2000) and MEGA 3.0 (Kumar, Tamura & Nei, 2004) for exploratory analyses of sequence variation. For each population of *M. coloradensis*, basic genetic diversity was calculated as the probability that two randomly chosen haplotypes are different (analogous to heterozygosity for diploid loci). All pairwise F_{ST} values were calculated, and analysis of molecular variation (AMOVA), as per Weir & Cockerham (1984), was implemented in Arlequin to assess the level and significance (using 100 000 permutations of the data) of spatial genetic structuring within versus among collection sites.

We tested for evidence of IBD, using a Mantel test in the program IBD (Bohonak, 2002) by regressing pairwise genetic distance (as Slatkin's linearised F_{ST} , Slatkin, 1995) against geographic distance. A positive relationship indicates IBD and is expected under conditions of limited ongoing gene flow among populations in migration–drift equilibrium; however, it breaks down under conditions of extreme dispersal limitation because of the overwhelming effect of local genetic drift against the lack of migration (Slatkin, 1993). Hence, if population genetic structure has been driven primarily by past allopatric fragmentation followed by a lack of gene flow, then significant IBD should not be detected.

We looked for further evidence of processes influencing population genetic structure with a nested clade analysis (NCA, Templeton, Boerwinkle & Sing, 1987). NCA was developed to distinguish between historical events (e.g. allopatric fragmentation) and ongoing ones (e.g. dispersal and gene flow) given current population genetic patterns. Specifically, NCA uses a coalescent approach to inform such inferences, based on a haplotype network broken into nested hierarchical clades and associated geographic information. Although the method has been questioned, mainly based on its inability to place statistical significance on results from an inference key (e.g. Knowles & Maddison, 2002), it has been implemented

in several phylogeographic studies and found to give results concordant with expectations based on local biogeographic history (e.g. DeChaine & Martin, 2004) and known population histories (e.g. Templeton, 2004).

We constructed a haplotype network using the statistical parsimony method described by Templeton, Crandall & Sing (1992) implemented in the software *rCS* 1.21 (Clement, Posada & Crandall, 2000). Networks are often more meaningful than bifurcating trees to show evolutionary relationships at the intraspecific level because of the concurrence of ancestral and derived haplotypes in extant populations (e.g. Posada & Crandall, 2001). We resolved reticulations (loops) in the network using the common theoretical prediction that singleton haplotypes are more likely to be connected to non-singletons than to other singletons or to unsampled haplotypes (Crandall & Templeton, 1993; Posada & Crandall, 2001).

For NCA, we delineated nested clades for the haplotype network according to rules given by Templeton *et al.* (1987). Given the topology of these clades and their proportional distribution among sample sites, the software *GEODIS* 2.4 (Posada, Crandall & Templeton, 2000) provides statistical tests of the correlation between phylogeny and geography. Rather than using geographic coordinates as *GEODIS* input, we created user-defined distances between sites specifically because of the close geographic proximity of two populations separated by a steep, high-elevation (>4200 m a.s.l.) ridgeline (see Fig. 2). In this case, geographic coordinates would suggest that these populations are much closer than they effectively are; they also occupy different major watersheds (defined by having eight-digit U.S. Geological Survey Hydrologic Unit Codes). Populations of *P. neomacropyga* on opposite sides of this type of topographic dispersal barrier are isolated, as are populations occupying different mountaintop islands (Finn *et al.*, 2006). To preserve relative degrees of pairwise effective distance between sites for *GEODIS* analysis, we defined all populations as equidistant, with the exception of those that shared neither a major watershed nor a mountaintop 'island', in which case a pair of populations was given a distance twice that of the other pairs. As such, geographical locations were simplified so that pairs of sites occupied one of two categories: near or far. Because 'true' effective distances among populations occupying a heterogeneous landscape are

difficult to infer (e.g. Wiens, 2001), simplified categorical classification is justified. Any nested clade for which the phylogeny–geography relationship was significant according to *GEODIS* ($P < 0.05$) was evaluated using the most recent inference key developed for NCA (Templeton, 2004).

Results

We found populations of *M. coloradensis* only at the outlet streams of four relatively large lakes (including the type locality; Table 1, Fig. 2b). These four populations were located in the headwaters of two major watersheds: two in the St Vrain and two in the Big Thompson. Three populations (NBC, GRL, CTL) occurred in extraordinarily high densities (the Medusa effect). The Chasm Lake population (CHM) had apparently lower densities but also was more difficult to sample because of stream flow through and below a large boulder field.

Sample sizes for genetic analyses were 47 (NBC), 21 (CHM), 53 (GRL) and 50 (CTL). We identified 26 total haplotypes across the four populations, and local genetic diversity ranged from 0.67 at CTL to 0.90 at CHM, which had the highest diversity despite having the smallest sample size (Table 2). Twenty-two of the 307 nucleotide sites were variable because of substitutions, and two of these were hypervariable. There were 20 transitions and four transversions, with three of the substitutions non-synonymous. As expected for insect mtDNA, nucleotide frequencies were strongly AT-biased (freq. T = 0.36, C = 0.20, A = 0.31, G = 0.13).

Several results indicated isolation among populations. First, all populations harboured private haplotypes (those found in only a single population); 21 of the 26 total haplotypes were private (see Table 2). Secondly, all pairwise F_{ST} values were highly significant ($P < 0.0001$). *AMOVA* suggested that differences among populations explained a significant amount of total genetic variation (17.3%, $P < 0.0001$) with the remaining proportion explained by within-population variation.

The haplotype network generated by *rCS* was relatively shallow, with at most five mutational steps separating haplotypes, and it demonstrated an unambiguous ancestral haplotype (no. 6) because of its abundance and interior position in the network (Fig. 3). The ancestral no. 6 was also the only haplo-

Table 1 Alpine lake outlets sampled for *Metacnephia coloradensis*, early August–early September 2003. For those sites where the species was present, the lake name is followed in parentheses by the three-letter code used for the site throughout the paper.

Alpine lake sampled	Drainage	Elev. (m)	Latitude (N)	Longitude (W)	Sample date	<i>M. coloradensis</i> present?
Unnamed	S. St. Vrain (main)	3426	40°03.719'	105°38.135'	1 Aug	No
Summit Lake	Bear Creek	3840	39°35.785'	105°38.212'	2 Aug	No
Pass Lake	N. Fork Snake R.	3551	39°39.267'	105°52.603'	2 Aug	No
Oliver Twist Lake	Mosquito Creek	3654	39°17.650'	106°09.819'	3 Aug	No
Crystal Lake (CTL)	Roaring River	3450	40°28.249'	105°38.750'	6 Aug	Yes
Unnamed	Hague Creek	3414	40°28.570'	105°40.761'	7 Aug	No
Unnamed	N. Fork Big Thompson	3486	40°29.745'	105°37.399'	12 Aug	No
Unnamed	N. Fork Big Thompson	3465	40°29.893'	105°37.375'	12 Aug	No
Lake Dunraven	N. Fork Big Thompson	3378	40°30.207'	105°37.294'	12 Aug	No
Lake Louise	N. Fork Big Thompson	3306	40°30.466'	105°37.017'	12 Aug	No
Green Lakes no. 3	N. Boulder Creek	3396	40°03.046'	105°36.834'	14 Aug	No
Green Lakes no. 4	N. Boulder Creek	3438	40°03.345'	105°37.040'	14 Aug	No
Green Lakes no. 5 (NBC)	N. Boulder Creek	3558	40°03.215'	105°37.618'	14 Aug	Yes
Frozen Lake	Glacier Creek	3477	40°15.568'	105°38.492'	15 Aug	No
Green Lake (GRL)	Glacier Creek	3462	40°15.320'	105°37.928'	15 Aug	Yes
Blue Lake	Glacier Creek	3345	40°16.034'	105°37.914'	15 Aug	No
Chasm Lake (CHM)	Roaring Fork	3537	40°15.564'	105°36.117'	16 Aug	Yes
Keplinger Lake	Hunters Creek	3506	40°14.496'	105°37.500'	18 Aug	No
Pipit Lake	Ouzel Creek	3423	40°11.520'	105°40.009'	21 Aug	No
Lake Powell	N. Inlet Creek	3465	40°15.397'	105°39.691'	22 Aug	No
Snowbank Lake	N. St. Vrain	3456	40°14.404'	105°38.586'	22 Aug	No
Lion Lake no. 2	N. St. Vrain	3429	40°14.246'	105°38.488'	22 Aug	No
Lake of Many Winds	N. St. Vrain	3489	40°13.447'	105°39.898'	23 Aug	No
Unnamed	S. St. Vrain (N. trib.)	3396	40°04.503'	105°37.432'	26 Aug	No
Blue Lake	Mitchell Creek	3387	40°05.266'	105°36.970'	27 Aug	No
Square Top Lakes	Duck Creek	3614	39°35.439'	105°44.298'	7 Sep	No

type found in all four populations. Only three haplotypes (including the ancestral) were found in both major watersheds (Fig. 3), suggesting that watershed boundaries might have been important dispersal barriers for this species.

Despite a high degree of population genetic structure, both a Mantel test of IBD and NCA suggested limited ongoing gene flow. The Mantel results suggested IBD based on a positive correlation between genetic and geographic distance ($P = 0.04$, Fig. 4), despite the small sample size of population pairs ($N = 6$). For NCA, a simplified diagram of the haplotype network (Fig. 3) is shown in Fig. 5, along with a delineation of clades. Because of the relatively shallow genetic structure, two-step clades were the most inclusive with the exception of the total cladogram level. Nonetheless, one one-step clade (1–4), both two-step clades and the total cladogram all produced significant results from GEODIS. Based on the inference key, both two-step (intermediate-sized) clades suggested ongoing restricted gene flow with

some long-distance dispersal. Restricted gene flow and IBD were inferred from the patterns in the smallest significant clade (1–4) and the total cladogram (see Table 3 for summary).

Discussion

Despite a survey of 26 potentially suitable locations, we found only three previously undiscovered populations of *M. coloradensis* in alpine areas in the vicinity of the type locality. The sparse distribution probably is attributable to habitat specificity of this species; all four populations occupied the outlets of large lakes well above treeline (>3450 m a.s.l.). Site characteristics such as relatively high lake productivity also are probably important for *M. coloradensis* (Adler *et al.*, 2004), although little research has been carried out to understand specific habitat requirements of this species.

Habitat specificity of stream insects can contribute to natal site fidelity and, therefore, to low levels of overland dispersal, leading to high levels of genetic

Table 2 Sample size (*n*), genetic diversity and frequency of 26 haplotypes at each of four Colorado sites occupied by *Metacnephia coloradensis*

Site	NBC	CHM	GRL	CTL
<i>n</i>	47	21	53	50
Diversity	0.80	0.90	0.68	0.67
Haplotype	Haplotype frequencies			
1	0.34		0.23	
2	0.26	0.10		
3	0.02			
4	0.09			
5	0.02			
6	0.15	0.19	0.51	0.50
7				0.04
8		0.05	0.04	0.22
9			0.15	
10			0.02	
11	0.04			
12				0.02
13				0.20
14		0.05		
15		0.24		
16		0.10		
17		0.05		
18			0.04	
19			0.02	
20				0.02
21	0.02			
22	0.02	0.14		
23		0.05		
24		0.05		
25	0.02			
26	0.02			

Shaded haplotypes are private.

structuring (Wishart & Hughes, 2001, 2003; Kelly, Rundle & Bilton, 2002; Finn *et al.*, 2006). The habitat specificity and sparse distribution of *M. coloradensis*, in addition to life-history traits that suggest limited dispersal ability (e.g. obligate autogeny and mating on the ground at the emergence site), strengthen the main hypothesis that populations should show a high degree of spatial genetic structure and reveal historical patterns of postglacial allopatric fragmentation.

Population genetic analyses demonstrated a significant degree of spatial structure in *M. coloradensis*. However, both a Mantel test and NCA implicated a limited amount of ongoing gene flow and long-distance dispersal. Such results suggest that current population genetic structure of *M. coloradensis* is driven primarily by ongoing processes and does not retain a strong signature of the pattern of postglacial allopatric fragmentation.

Furthermore, populations of the more widespread alpine black fly *P. neomacropyga* had a higher degree of geographic structure than those of *M. coloradensis* (Table 4). Overall F_{ST} for *P. neomacropyga* based on the same mtDNA fragment was more than twice that for *M. coloradensis*. If a higher value of F_{ST} alone indicates lower dispersal among populations, an inference that has been supported by several studies directly comparing species biology to population genetic patterns (Bohonak, 1999; Miller, Blinn & Keim, 2002; Wilcock, Nichols & Hildrew, 2003), then successful between-

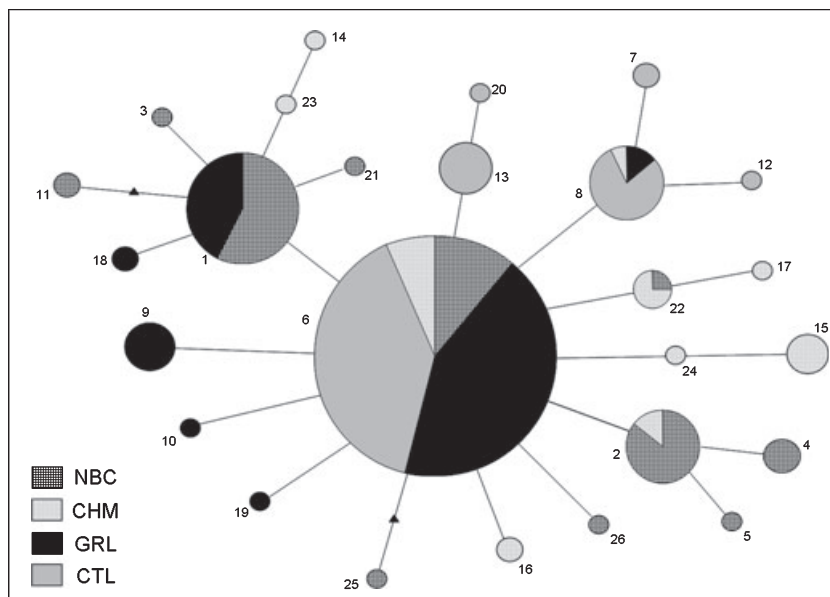


Fig. 3 Haplotype network indicating proportional representation of each haplotype (circle) in each of four populations (indicated by colour/fill pattern) of *Metacnephia coloradensis*. Solid fill represents haplotypes in Big Thompson watershed; hatched fill indicates St Vrain watershed (see Fig. 2). Each line is one mutational change. Size of circle is proportional to total number of individuals possessing each haplotype. Small triangles represent hypothesised unsampled haplotypes. Numbers are haplotype numbers as in Table 2, and haplotype 6 is the unambiguous ancestral haplotype.

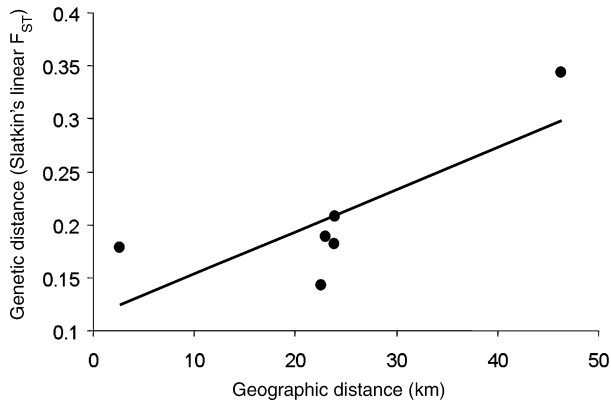


Fig. 4 Genetic distance (as Slatkin's linearised F_{ST}) versus geographic distance. Each point represents a unique pair of populations. Slope = 0.0051; $P = 0.04$ (Mantel test).

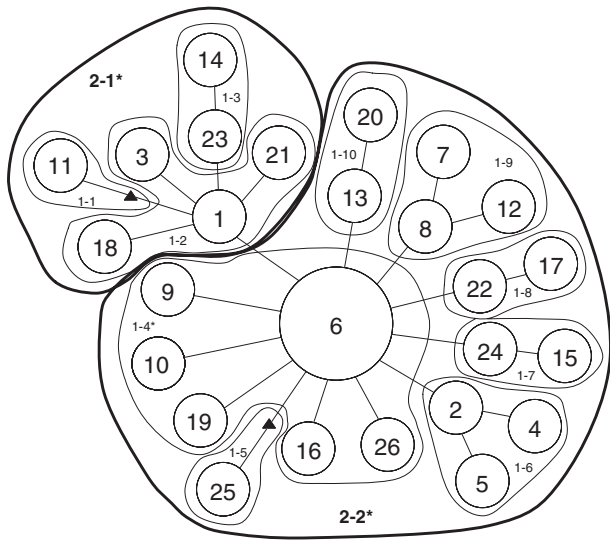


Fig. 5 Simplified haplotype network for *Metacnephia coloradensis*, from Fig. 3, with clade nestings used in nested clade analysis. Thin lines enclose one-step clades; thick lines enclose two-step clades. Stars indicate geographically significant clades according to GEODIS.

stream dispersal appears to be occurring significantly more often in *M. coloradensis* than in *P. neomacropyga*.

This conclusion, however, assumes near-equal effective population sizes (N_e) in both species, as larger N_e will negatively influence F_{ST} (Wright, 1951). Table 4 compares several other population genetic statistics for *M. coloradensis* and *P. neomacropyga*, whose populations are distributed across overlapping spatial extents. Mean local genetic diversity is significantly higher for *M. coloradensis*, and we identified the same number of COI haplotypes in each species,

Table 3 Results of nested clade analysis for *Metacnephia coloradensis*. Only clades with significant geographical relationships are included.

Nested clade	Inference key steps	Conclusion
1-4	1,2,3,4, no	Restricted gene flow with IBD
2-1	1,2,3,5,6,7, yes	Restricted gene flow with some long-distance dispersal
2-2	1,2,3,5,6,7, yes	Restricted gene flow with some long-distance dispersal
Total cladogram	1,2,3,4, no	Restricted gene flow with IBD

IBD, isolation by distance; refer to Fig. 5 for clade topology.

despite many fewer (4 versus 11) *M. coloradensis* local populations. These observations imply that, despite its sparse distribution across the landscape, this species has maintained significant genetic diversity. The readily-observable local success of populations in terms of density of individuals (the Medusa effect) suggests large local N_e , which would help explain these patterns. In populations of larger effective size, lower-frequency haplotypes are less likely to drift to extinction. *Prosimulium neomacropyga*, conversely, maintains relatively much lower local population densities in the study area, with well-spaced larvae occupying only the downstream surfaces of some large cobbles and boulders (D.S. Finn, personal observation).

Because of slow genetic drift, large populations are less likely than their smaller counterparts to have achieved migration-drift equilibrium since historical fragmentation events, especially when gene flow is limited (e.g. Neigel, 1997), as inferred in the current study populations. Time since isolation of *M. coloradensis* on mountaintop islands likely dates back only to the late Pleistocene, c. 10 000 years BP (see also Finn *et al.*, 2006). A large local N_e combined with comparatively recent fragmentation can cause current population structure to retain a signature of historical patterns. This historical imprint will not only affect basic population statistics such as F_{ST} , but also will influence the conclusions of more complex phylogeographic analyses, including IBD tests (Slatkin, 1993; Garnier *et al.*, 2004) and NCA (Knowles & Maddison, 2002; Templeton, 2004). Conclusions drawn from these analyses may be indicative of pre-fragmentation rather than current processes.

Table 4 Population genetic statistics for two autogenous, alpine black flies distributed across overlapping spatial extents in northern Colorado. Results for both species are based on the same molecular marker; all statistics for *Prosimulium neomacropyga* have been previously reported by Finn *et al.* (2006).

Species	Habitat	No. streams occupied	No. COI haplotypes	No. private haplotypes	F_{ST}	Mean genetic diversity (SD)	IBD slope (P -value)
<i>Metacnephia coloradensis</i>	Lake outlets	4	26	21	0.17	0.76 (0.11)	0.0051 (0.04)
<i>Prosimulium neomacropyga</i>	Lake outlets and other streams	11	26	16	0.38	0.55 (0.16)	0.0068 (0.04)

COI, cytochrome oxidase I; IBD, isolation by distance.

Metacnephia coloradensis might once have consisted of a series of populations that were more widespread across cooler Pleistocene habitats. Large lakes that currently exist at lower altitudes, as well as glacial-period lakes that are no longer extant (Elias, 1996), might have provided ideal outlet habitat for *M. coloradensis*. Limited gene flow among adjacent populations would have been more likely in the absence of strong isolation on mountaintop islands. During the warming period, some populations persisted, gradually retreating to the highest altitudes where new lakes appeared following glacial recession. Although current gene flow among these isolated habitats is unlikely, extremely large local populations would have decreased drift rates allowing retention of the historic signal of limited gene flow.

The haplotype network suggests some influence of major watershed boundaries as barriers to gene flow. These divides extend to altitudes significantly lower than the terminal moraines of Pleistocene glaciers in Rocky Mountain National Park (Elias, 1996) and are likely to have restricted dispersal among aquatic populations even when climates were cooler and current alpine-restricted species were more widespread. Based on population genetic patterns of *P. neomacropyga* (Finn *et al.*, 2006), alpine populations that are closer to migration–drift equilibrium are not expected to be spatially structured according to major watershed; however, this pattern might be expected if large *M. coloradensis* populations have retained historical patterns. Watershed boundaries have been shown to restrict current gene flow in other stream insects (e.g. Hughes *et al.*, 1999; Wishart & Hughes, 2001, 2003; Hughes, Hillyer & Bunn, 2003).

Further comparative studies of *M. coloradensis* and *P. neomacropyga* are called for because these species appear to share similar phylogeographic histories across a much broader spatial scale. *Prosimulium*

neomacropyga in Colorado is at the extreme southern periphery of its range; its distribution is centred in Beringia (region spanning the Bering strait, from the Kolyma River in Siberia to the Mackenzie River in Canada). The closely related sister species of *M. coloradensis* is *M. sommermanae* (Stone), which has a distribution similar to that of *P. neomacropyga*, except it does not extend south to the patchily distributed alpine habitats in the southern Rocky Mountains. *Metacnephia coloradensis* is found here instead and has diverged enough from *M. sommermanae*, possibly in isolation, to now be recognised as a separate species. The two species differ chromosomally by a single inversion that is fixed in *M. sommermanae* but linked to the Y chromosome in *M. coloradensis* (Adler *et al.*, 2004), and morphologically in characteristics of the eyes (Peterson & Kondratieff, 1995). Similar distributions and presumably similar biological traits probably led to similar responses to glacial advances and retreats in *P. neomacropyga* and *M. sommermanae*. A question is whether these two species left isolated southern populations in response to the same glacial episode. A comparative study of neutral population genetic structure across the entire range would provide clues.

Metacnephia coloradensis has two of three important attributes leading to rarity (Rabinowitz, 1981): narrow habitat tolerance and restricted geographic range. It does not have the third, small local population size. Just one of the three, however, is sufficient to produce rarity and possible conservation concern (Rabinowitz, 1981). Under the current strategy for assessment of conservation concern by the US Natural Heritage Program (Stein & Flack, 1997), *M. coloradensis* would receive the lowest rank (G1, or 'critical impairment') for species still occupying known locations. Faced with the potential of continued broad-scale disturbance such as climate change and atmospheric

deposition (e.g. see Baron *et al.*, 2000; Welker, Bowman & Seastedt, 2001), *M. coloradensis* will likely have little capacity to respond. An understanding of the specific environmental characteristics influencing current and past distribution will be necessary to predict the response of this and other alpine-stream species to changing conditions.

Acknowledgments

Special thanks to Bill Black and his lab at Colorado State University for provision of space and equipment and help with molecular methods; and to Kristy Duran, with whom DSF first experienced the 'Medusa' effect, and Jeremy Monroe, who took pictures of it. Folks at the CU Mountain Research Station and INSTAAR (especially Bill Bowman and Nel Caine) helped obtain access to the North Boulder Creek watershed, and Rocky Mountain National Park provided collecting permits. Boris Kondratieff was contagiously enthusiastic about all insects that we ever collected. N. LeRoy Poff provided sound mentorship through the graduate career of DSF. He, Bill Black, and two anonymous reviewers provided useful feedback on earlier versions of the manuscript. DSF was supported by an EPA-STAR graduate fellowship.

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- (Manuscript accepted 30 August 2006)