Freshwater Biology (2003) **48**, 2149–2162

Genetic structure in a montane mayfly *Baetis bicaudatus* (Ephemeroptera: Baetidae), from the Rocky Mountains, Colorado

JANE M. HUGHES*, PETER B. MATHER†, MIA J. HILLYER*, CATH CLEARY* AND BARBARA PECKARSKY‡

*Cooperative Research Centre for Freshwater Ecology, Australian School of Environmental Studies, Griffith University, Nathan, Queensland, Australia

†School of Natural Resource Sciences, Queensland University of Technology, Gardens Point, Brisbane, Queensland, Australia

‡Rocky Mountain Biological Laboratory, Crested Butte, Colorado, U.S.A. and Department of Entomology, Cornell University, Ithaca, NY, U.S.A.

SUMMARY

1. Populations of a number of sub-tropical stream insect species have been found to show unexpected patterns of genetic variation, with more differences between samples from the same stream than between whole streams or between subcatchments. Many samples also showed deviations from Hardy–Weinberg proportions. It has been proposed that these patterns result from limited numbers of matings contributing to a given stream reach, because adults emerge throughout the year, and low levels of larval drift between reaches. These patterns may be less likely in a northern hemisphere montane species with synchronous emergence of adults and high levels of drift. We tested the hypothesis that patterns of genetic variation in a montane mayfly from the Rocky Mountains, Colorado, would reflect a pattern of 'isolation by distance' with samples from the same creek being more similar than samples from different creeks and that deviations from Hardy–Weinberg proportions would be minimal.

2. Based on allozyme variation, the hypothesis of minimal deviations from Hardy–Weinberg proportions was not supported and there was no evidence of isolation by distance. Nevertheless the levels of differentiation among samples from within the same stream were less than those reported for most subtropical species.

3. Results from analysis of a fragment of the cytochrome oxidase gene (subunit 1) revealed contrasting patterns. The levels of genetic differentiation were an order of magnitude higher between streams than among samples within streams. In addition, although there was no significant isolation by distance effect overall, a nested clade analysis provided evidence for restricted gene flow with isolation by distance for some clades.

4. We suggest that these contrasting results may reflect the differences in male and female dispersal patterns. While differentiation at nuclear gene markers (allozymes) give information about both male and female dispersal, mitochondrial DNA markers reflect only female dispersal. We suggest that in this species, female dispersal may be more restricted, perhaps mostly along stream channels, whereas male dispersal is

Correspondence: Jane M. Hughes, Cooperative Research Centre for Freshwater Ecology, Australian School of Environmental Studies, Griffith University, Nathan, Queensland, 4111, Australia. E-mail: jane.hughes@mailbox.gu.edu.au

more widespread. An alternative explanation for the different results is the different evolutionary rates of the mitochondrial and nuclear markers.

Keywords: allozymes, genetic structure, mayfly, mtDNA

Introduction

Genetic techniques have been widely used to investigate patterns of dispersal in a range of plant and animal species. Regardless of the particular marker employed, all methods rely on the idea that if there is widespread dispersal among subpopulations of a species, levels of genetic differentiation will be low, whereas if dispersal is restricted, either by physical barriers or innate limitations to mobility, then genetic differentiation is likely to be high (Slatkin, 1985).

In stream invertebrates, the levels of genetic differentiation among catchments, and even among different streams in the same catchment, can be very high in species that are restricted to movement within the stream channel. For example, a number of species of freshwater shrimps show high levels of genetic differentiation among nearby populations, based both on allozymes and mitochondrial DNA (mtDNA) analysis (Hughes *et al*., 1995, 1996; Hurwood & Hughes, 2001; Hurwood *et al*., 2003). Similar patterns have been observed in insects with limited powers of flight, such as blephariceridid midges (Wishart & Hughes, 2001, 2003) and those that are separated by significant geographical barriers, such as montane stoneflies (Hughes *et al*., 1999).

Most insects however show low levels of genetic differentiation among populations both within and among catchments, an observation that has been attributed to extensive dispersal resulting from adult flight (Sweeney, Funk & Vannote, 1987; Schmidt, Hughes & Bunn, 1995; Hughes *et al*., 1998, 2000; Miller, Blinn & Keim, 2002; Monaghan *et al*., 2002). Many of these studies used allozyme variation to assess the extent of genetic differentiation, and many observed levels of deviation from Hardy–Weinberg proportions that were higher than would be expected by chance (e.g. Schmidt *et al*., 1995; Hughes *et al*., 1998, 2000; Monaghan *et al*., 2002; Hughes, Hillyer & Bunn, 2003). Studies that have focussed on subtropical trichopteran and ephemeropteran species in eastern Australia also reported as much or more genetic differentiation among populations in separate reaches of individual streams than between streams or even between catchments (e.g. Hughes *et al*., 1998, 2000, 2003).

One explanation proposed to explain these unexpected patterns of genetic variation was that insect larvae sampled in a particular reach of a stream may represent the offspring of only a limited number of matings, and that subsequent mixing of larvae may be minimal because of very low levels of larval drift in these streams (Kerby, Bunn & Hughes, 1995). The fact that deviations from Hardy–Weinberg proportions appeared to occur randomly across sites and loci, and patterns seen among reaches within a stream differed among loci provided some evidence to support this hypothesis (Hughes *et al*., 1998, 2000). Furthermore, this explanation is plausible for mayfly species in subtropical eastern Australia, which emerge asynchronously all year round; and thus larvae in a particular reach at a particular time may represent the offspring of only a few females (Schmidt *et al*., 1995; Hughes *et al*., 2000). In contrast, this pattern may not be typical of species with synchronous emergence of adults and large mating swarms, which is more typical of mayfly species in the northern hemisphere (Edmunds, Jensen & Berner, 1976; Harker, 1992).

We predicted that in northern hemisphere montane species with synchronous patterns of adult emergence, we would observe low numbers of Hardy–Weinberg deviations coupled with large genetic differences among populations at the largest scale (Hughes *et al*., 1999). This prediction was supported by genetic data from a small species of stonefly, *Yoraperla brevis*, in the Rocky Mountains in Montana (Hughes *et al*., 1999), which showed limited deviations from Hardy–Weinberg proportions, and highest levels of genetic differentiation among different streams. In fact, levels of differentiation between streams were so high that they suggested that this species rarely disperses between streams at all. Therefore, the prediction needs to be tested in a montane northern hemisphere species with similar flight abilities to those examined in subtropical Australia.

In this study, we examined allozyme and mtDNA variation in a baetid mayfly *Baetis bicaudatus* from streams in the Rocky Mountains, Colorado. *Baetis bicaudatus* adults emerge from a particular stream reach relatively synchronously compared with Australian baetids (Peckarsky, Taylor & Caudill, 2000). Recent observations, coupled with allozyme and DNA analysis have revealed that *B. bicaudatus* is sympatric with a number of other *Baetis* species, one of which may be an undescribed cryptic species that emerges later in the summer than *B. bicaudatus*, but also relatively synchronously (B. Peckarsky and J. M. Hughes, unpublished data.). Mating swarms of adults are seen in the vicinity of streams, but often swarm considerable distances from the stream edge (Peckarsky *et al.*, 2002), suggesting the potential for cross-catchment adult dispersal. *Baetis bicaudatus* is thus an appropriate species in which to test the hypotheses that in a synchronously emerging species:

(i) genetic samples will not deviate from Hardy–Weinberg proportions more often than expected by chance,

(ii) genetic differentiation among samples from different streams and sites within the same stream will be non-significant (as a result of extensive dispersal) or

(iii) if there is significant differentiation, it will be at the largest spatial scale, among streams.

We analysed samples using both allozymes, which allow tests for deviations from Hardy–Weinberg proportions and comparisons with previous studies, and mtDNA. mtDNA, because it is haploid and maternally inherited, has a four-fold smaller effective population size than nuclear DNA (Birky, Fuerst & Maruyama, 1984) and thus should be more sensitive to any limited gene flow that may occur.

An alternative explanation put forward by Monaghan *et al.* (2002) to explain the genetic patterns seen in many stream insect species is that they result from recent population expansions into an area and that subpopulations have not been there long enough to have reached equilibrium between gene flow and genetic drift. Thus, we used nested clade analysis, which uses a statistical framework to examine possible contemporary and historical processes that could cause significant geographical relationships among haplotypes (Templeton, 1998).

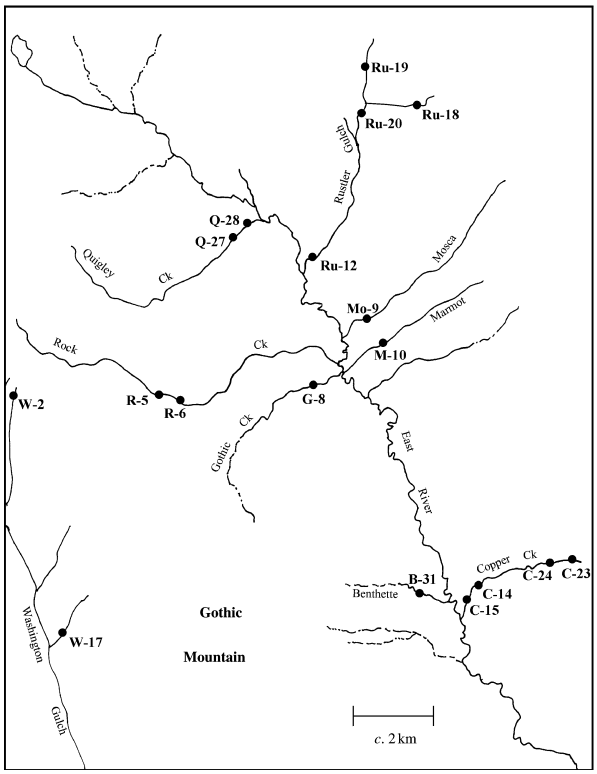

Fig. 1 Sampling sites for *Baetis bicaudatus* from the Rocky Mountains, Colorado. Site names and abbreviations are Rustlers Gulch (Ru); Copper Creek (C); Rock Creek (R); Quigley Creek (Q); Washington Gulch (W); Gothic (G); Mosca (Mo); Marmot (M) and Benthette (B).

Methods

Sampling design

Baetis bicaudatus overwinters as first instar larvae, which begin growth and development as the snow melts and floodwaters recede in May and early June in these high altitude streams. Larvae become mature in late June, throughout July, and into early August, at which time they emerge to the adult stages, mate and oviposit within 3 days of emergence. (Peckarsky *et al.*, 2001) The specimens collected for this project were late instar larvae. Samples were collected from a number of streams flowing into the Upper East River near the Rocky Mountain Biological Laboratory (Fig. 1) as well as sites on the west side of Gothic Mountain, which flow into the East river much further downstream. Our initial design involved collecting equal numbers of *Baetis* individuals from multiple sites in multiple streams to compare genetic variation among sites

within a stream to that observed among streams. However, subsequent allozyme analysis revealed the presence of a cryptic species, which resulted in an unbalanced sampling design and variable sample sizes. The final sampling scheme thus consisted of 16 sites, from eight streams flowing into the East River on the east side of Gothic Mountain, and two from streams flowing off the west side of Gothic Mountain (Fig. 1). Multiple samples were collected from Rustler's Gulch (four sites), Copper Creek (four sites), Rock Creek (two sites), Quigley Creek (two sites) and Washington Gulch (two sites).

Baetis larvae were collected by agitating rocks upstream of a D-net to catch all dislodged invertebrates. Samples were sorted in a plastic dish beside the stream and all *Baetis* were removed and kept cool in a small ice bag until they could be frozen in liquid nitrogen. They were stored in liquid nitrogen until the day they were transported back to Australia, when they were transferred to dry ice and kept frozen before being moved to a −80 °C freezer, where they were kept until genetic analysis.

Allozyme analysis

We used cellulose acetate electrophoresis (Helena Laboratories, Titan III plates; Beaumont, TX, USA) methods similar to those described by Schmidt *et al.* (1995) and stains modified from Richardson, Baverstock & Adams (1986). Twenty-five enzymes were screened for variation and five were chosen that showed variation and could be reliably interpreted. These were glucose phosphate isomerase (PGI, IECC no. 5.3.1.9), phosphglucomutase (PGM, IECC no. 2.7.5.1), amino aspartate transferase (AAT, IECC no. 2.6.1.1), peptidase (PEPB and PEPC, IECC no. 3.4.11). All were encoded by single loci, except AAT, which had two loci, both of which were variable.

Mitochondrial DNA analysis

Total genomic DNA was extracted from each individual using a modification of several Chelex protocols (Singer-Sam, Tanguay & Riggs, 1989; Walsh, Metzger & Higuchi, 1991; Sweet *et al.*, 1996). The residual tissue from allozyme analysis was transferred to clean 1.5 mL eppendorf tubes containing 500 μL of 5% chelex solution (Bio-Rad, Hercules, CA, USA) and ground using a plastic micro-pestle. Seven microlitres of proteinase K (20 mg mL^{-1}) were added before samples were vortexed and incubated at 54 °C overnight. Prior to polymerase chain reaction (PCR), samples were heated at 96 °C for 20 min, and centrifuged at 16 000 *g* for 2 min at room temperature.

The primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) were used to amplify approximately 710 bp fragment of the cytochrome oxidase subunit 1 gene (COI). Reactions contained 1 μL of 10 mM dNTP's (Bioline, London, UK), 5 μL of 10 x polymerase reaction buffer, 4 μL 50 mM $MgCl_2$, 1 unit of *Taq* polymerase (all Invitrogen, Carlsbad, CA, USA), 2 μL of each primer (10 μM), 1 μL (10–90 ng) of DNA template, and adjusted to a final volume of 50 μL with ddH_2O. The PCR temperature profile consisted of a 5 min denaturing period at 94 °C, then 15 cycles of 94 °C denaturing for 30 s, 40 °C annealing for 30 s and 72 °C extension for 1 min, followed by 25 cycles where annealing was increased to 55 °C. A final extension step of 72 °C for 5 min was followed by an indefinite hold period at 4 °C.

Prior to sequencing, purification of PCR product was performed using a QIAGEN QIAquick Gel Extraction kit as per the manufacturer's instructions. Concentrations of purified DNA were estimated by running 2 μL of product on a 1.6% agarose gel alongside a known marker. Sequencing reactions contained approximately 30 ng of clean DNA template, 3.2 pmol of primer, 4 μL of dye terminator mix (PE Applied Biosystems, Foster City, CA, USA) adjusted to a volume of 10 μL with ddH_2O. These were subjected to: 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. DNA was cleaned as per manufacturer's instructions and sequenced on PE Applied Biosystems 377 automated sequencer.

Statistical analysis

Allozymes. All samples were tested for deviations from Hardy–Weinberg proportions, using the exact test in GENEPOP (Raymond & Rousset, 1995). F_{IS}-values were calculated for all samples and loci in GENEPOP. Bonferroni corrections were used to account for multiple tests. F_{ST}-values were tested at different hierarchical levels using BIOSYS 1.7 and also for each stream separately, in the manner of Rank (1992). The levels in the hierarchy were 'among streams', 'among sites within streams' and 'within streams'. For hierarchical analysis, only those streams

that had multiple sites were included in the analysis, because for streams with single sites, it would not be possible to distinguish between real differences between streams or apparent differences between streams because no replicate sites had been sampled.

Mitochondrial DNA. Sequences were aligned in Bioedit (Hall, 1999) and a 520 bp fragment was retained for analysis. For the mtDNA data, analysis of molecular variance (AMOVA, in Arlequin, Schneider *et al*., 1997) was used to test the significance of the different levels of the hierarchy. Again, only those streams with multiple sites within them were included. In addition, individual AMOVA's were performed for each stream. The AMOVA's were performed using frequency data alone (F_{ST}) and incorporating sequence divergence using pairwise differences (Φ_{ST}). A Mantel's test was used to test for isolation by distance, using Slatkin's linearised F_{ST} against geographical distance (i.e. if dispersal is by flight) and stream distance (if dispersal is along the stream channel).

We used nested clade analysis to assess the contemporary and historical processes that could be responsible for the observed patterns of mtDNA variation. We used the TCS programme (Clement, Posada & Crandall, 2000), to create a network showing relationships among haplotypes. This method uses maximum parsimony to obtain the 95% plausible set of alternative networks. The nesting of clades was performed using the rules set out in Templeton *et al*. (1992) and Crandall (1996). GEODIS Version 2.0 (Posada, Crandall & Templeton, 2000) was used first to test for geographical association of haplotypes and clades at each nesting level, using contingency tests. Secondly, we calculated clade distance (D_c), which measures the geographical dispersion of that clade, and nested clade distance (D_n), which measures the mean distance of a clade from the geographical centre of the next higher nesting clade, i.e. the displacement of the clade in relation to all other clades within the same nested clade. Also, the average interior distance minus the average tip distance was estimated for each clade and for each nesting clade. Each of these D_c and D_n-values was tested for significance using 1000 permutations. The updated inference key (based on Templeton, Routman & Phillips, 1995) was used to infer the processes responsible for all haplotypes and clades that showed significant geographical relationships as indicated from the contingency analyses.

To test the hypothesis of a recent population expansion, a mismatch distribution was calculated using DnaSP (Rozas & Rozas, 1999). This method

Table 1 Results of tests for deviations from Hardy–Weinberg (F_{IS}-values given). F_{IS} measured using Weir and Cockerham's method: (+) heterozygote deficiency; (−) heterozygote excess

Site	*Aat-1*	*Aat-2*	*Pgi*	*Pgm*	*Pep-B*	*Pep-C*	Average *n* per locus
Marmot 10	–	–	+0.260*	−0.068	+0.215	–	55
Benthette 31	–	–	+0.041	−0.296	+0.162	–	30
Copper 14	–	–	–	−0.077	−0.233	–	15
Copper 15	–	–	+0.383*	−0.123	+0.183	–	29
Copper 23	−0.037	+0.664*	+0.142	+0.279	+0.113*	–	41
Copper 24	–	–	−0.021	−0.350	+0.006	–	13
Gothic 8	−0.026	–	−0.102	−0.340	−0.116*	–	21
Mosca 9	–	–	+0.319	+0.010	+0.002	−0.045	24
Quigley 27	−0.045	−0.014	−0.067	−0.086	−0.064	–	36
Quigley 28	−0.014	–	+0.654	+0.254	+0.322*	–	18
Rock 5	+0.378	–	+0.090	+0.198	+0.068	–	50
Rock 6	−0.010	–	+0.108	−0.174	+0.181	–	53
Rustlers 12	–	–0.029	–	+0.632	+0.044	–	10
Rustlers 18	−0.005	–	+0.031	−0.071	+0.182*	–	51
Rustlers 19	–	+0.662*	+0.319*	+0.148	+0.179*	–	48
Rustlers 20	−0.012	–	+0.001	+0.216	+0.243*	–	72
Washington 2	–	–	+0.192	+0.156	+0.216*	–	28
Washington 17	–	–	+1.000*	+0.025	+0.505*	–	20

*$P < 0.05$.

Table 2 F_{ST}-values for six allozyme loci, all loci combined, and all loci combined (excluding *Pep-B*): (i) for all streams; (ii) for streams with multiple sites only (hierarchical analysis); and (iii) within individual streams

	LOCUS						Combined across loci			Combined across loci (Ex. *Pep-B*)		
	Aat-1	*Aat-2*	*Pgi*	*Pgm*	*Pep-B*	*Pep-C*						
All streams												
Among all sites	0.008	0.008	0.010	0.013	0.071	0.019	0.040			0.012		
Streams with multiple sites only												
Among streams	0.002	−0.005	−0.005	−0.002	−0.023	−0.005	−0.013			−0.003		
Among sites within each stream	0.005	0.010	0.012	0.012	0.077	0.002	0.044			0.011		
Among all sites	0.007	0.005	0.007	0.010	0.056	−0.002	0.031			0.009		
Within individual streams							Mean (all loci)	Jackknife		Mean (Ex. *Pep-B*)	Jackknife	
								Mean	SD		Mean	SD
Copper Creek	0.0035	−0.0184	0.0067	0.0147	0.1000***	0.0052	0.0541	0.0667	0.04379	0.0098	0.0135	0.00709
Quigley Creek	0.0018	0.0015	−0.0098	−0.0118	0.1218***	0.0000	0.0519	0.0667	0.06167	−0.0089	−0.0106	0.00386
Rock Creek	0.0011	0.0000	−0.0039	−0.0083	0.0596***	0.0000	0.0256	0.0330	0.03361	−0.0064	−0.0083	0.00346
Rustlers Gulch	0.0018	0.0250**	0.0083	0.0032	0.0362***	−0.0060	0.0205	0.0243	0.01540	0.0055	0.0041	0.00334
Washington Gulch	0.0000	0.0000	0.0109	0.0422*	0.0715***	−0.0072	0.0519	0.0572	0.01864	0.0330	0.0453	0.02152

*$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$

compares the observed distances of pairwise nuclear differences between haplotypes to that expected under the sudden expansion model of Rogers & Harpending (1992). Time since expansion was calculated using the formula $\tau = 2ut$; where u is the mutational rate per sequence per generation, and t is the time in generations (Rogers & Harpending, 1992).

Results

Allozymes

Fourteen of 65 tests (21%) for Hardy–Weinberg were significant (Table 1), eight of which were at the *PepB* locus. This number of deviations was greater than expected from chance, although most

Table 3 Frequency of each cytochrome oxidase subunit 1 gene (COI) haplotype detected at the 18 sample sites

	M-10	B-31	C-14	C-15	C-23	C-24	G-8	Mo-9	Q-27	Q-28	R-5	R-6	Ru-12	Ru-18	Ru-19	Ru-20	W-2	W-17
HAP-1	1	2	1	4	4	1	3	1	3	2	5	2	1		2	5	9	2
HAP-2			8								1						3	
HAP-3	2			2	1	1		1	2				1	1	4			
HAP-4		1		1	3	1			1	2	1		2		2		2	
HAP-5		2		1	2	2		2		1			1		2	1		
HAP-6		2		1		1	1		2	1			1	1	1	2		2
HAP-7											3	1					1	
HAP-8			1				3		8	4	4	5			2	2	2	
HAP-9					2	1												
HAP-10	9	8	1	6	3	3	2	11	1	2				2		5		1
HAP-11																1		
HAP-12				1														
HAP-13						1												
HAP-14														1				
HAP-15				1														
HAP-16																1		
HAP-17										1								
HAP-18											1							
HAP-19											1							
HAP-20						1												
HAP-21												1						
HAP-22		1																
HAP-23							1											
HAP-24							4											

Table 4 Results of AMOVA based on 520 bp of COI sequence data showing variation (i) for all streams, (ii) for streams with multiple sites only (hierarchical analysis) and (iii) within individual streams

	All populations		Minus copper 14	
	Φ_{ST}	F_{ST}	Φ_{ST}	F_{ST}
All streams				
Among all sites	0.2137***	0.1469***	0.1963***	0.1280***
Streams with multiple sites only				
Among streams	0.1213**	0.0528**	0.1518***	0.0749***
Among sites within each stream	0.0826***	0.0663***	0.0220	0.0157
Among all sites	0.1938***	0.1156***	0.1705***	0.0894***
Within individual streams				
Copper Creek	0.1166 ***	0.1147 ***	−0.0254	−0.0316
Quigley Creek	−0.0271	−0.0238		
Rock Creek	0.0915	0.0695		
Rustlers Gulch	0.0391	0.0302		
Washington Gulch	0.1382 *	0.0608		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

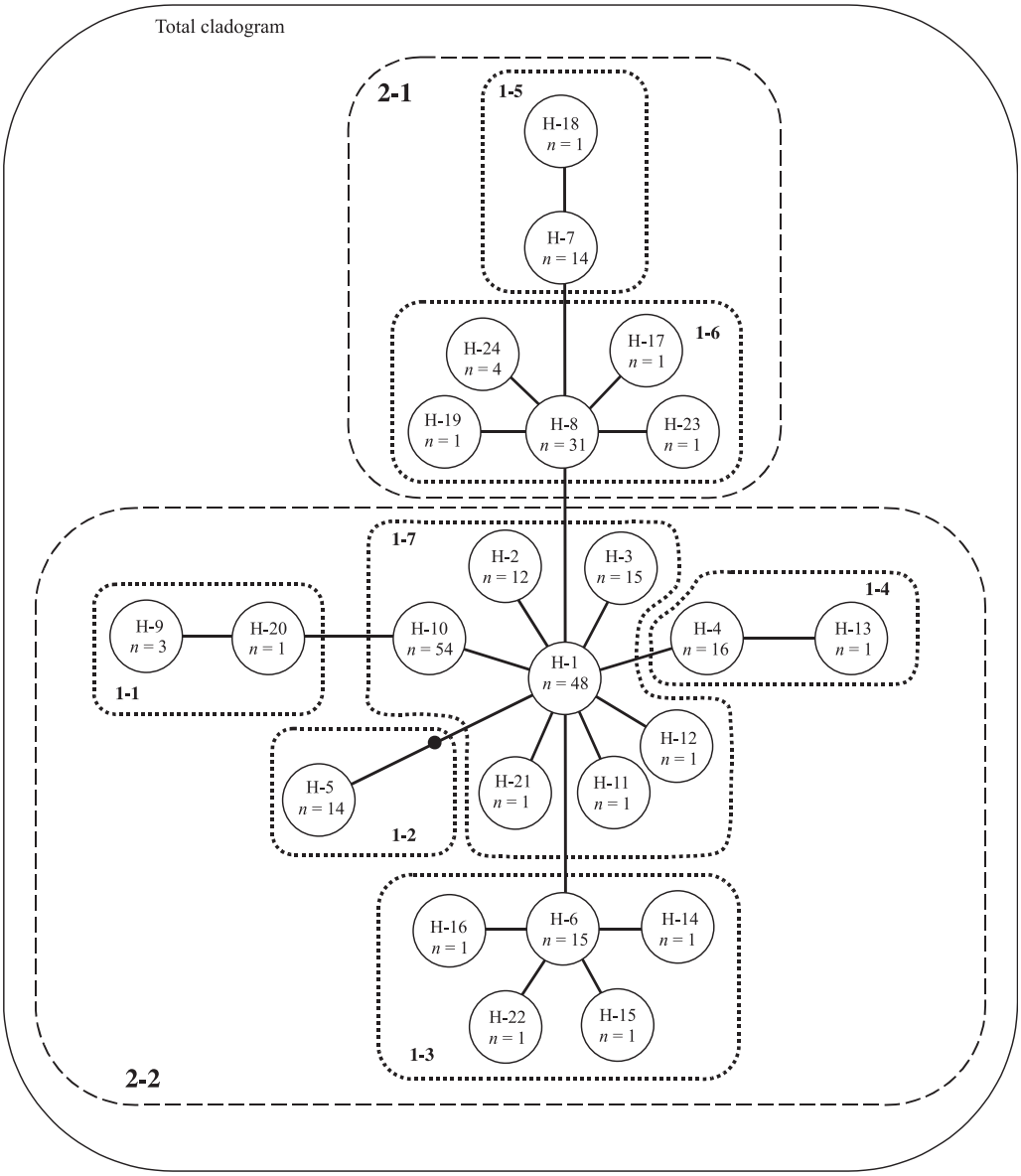

Fig. 2 Nested haplotype cladogram for *Baetis bicaudatus* with 95% plausible set of haplotype connections. Haplotypes H-1 to H-24 are shown with respective sample sizes (*n*) given. A solid branch between haplotypes indicates a single mutation. Haplotypes not detected in the sample are indicated by a solid black circle.

of the deviations were relatively small because, after Bonferroni correction, none were significant. Again contrary to expectations, for all six polymorphic loci, the F_{ST}-value among sites within streams was greater than the value among streams. F_{ST}-values were highly significant within all streams at the *PepB* locus and were also significant at *Aat-2* in Rustler's Gulch and *Pgm* in Washington Gulch (Table 2). In all streams except Rock Creek the jackknife mean F_{ST}-value was greater than the SD. Mantel's tests for isolation by distance, both aerial and stream distance were non-significant ($P > 0.05$), which indicates that there is no evidence for increasing genetic differentiation at larger spatial scales. Because *PepB* deviated from Hardy–Weinberg proportions and because F_{ST}-values were greatest for this locus, the data was reanalysed with *PepB* omitted. This resulted in the overall values of F_{ST} being reduced, but the trend remained the same, with no evidence of significant differentiation among streams and higher F_{ST}-values among sites within streams than among streams.

Mitochondrial DNA

From the 238 individuals analysed 24 unique haplotypes were observed (Table 3) with 19 variable sites (Appendix I). The transition/transversion ratio was 8 : 1 with 16 of the variable sites occurring at the third position and the remaining three at the first position. The sequences have been deposited in GenBank Accession numbers AY383574–AY383597.

The AMOVA indicated highly significant Φ_{ST} and F_{ST}-values among streams (Table 4). Both statistics were also highly significant for variation among sites within streams, although when each stream was analysed separately, only Copper Creek showed highly significant differences among sites within a stream. One site, Copper 14, had haplotype frequencies vastly different from all other sites in the same stream, even Copper 15, which was only 200 m downstream, but separated from Copper 14 by a 25-m waterfall. When Copper 14 was removed from the analysis, the variation among sites within streams was non-significant. Mantel's tests for isolation by distance were non-significant ($P > 0.05$), both for geographical (aerial) distance and for stream distance.

The haplotype network showed a well-resolved pattern with only one missing haplotype (Fig. 2). Generally haplotypes central to the network were widespread geographically, although some tip haplotypes also occurred in a number of streams, suggesting that adult dispersal may be widespread. The nesting design, based on the network is shown in Fig. 2. There were seven one-step clades, two, two-step clades and the total cladogram. The contingency analyses indicated three significant results (Table 5), the one-step clade, 1-7, the two-step clade, 2-1 and the total cladogram, which indicates that there was significant geographical structure of those clades.

For each of these clades, by following the inference key, restricted gene flow with isolation by distance is the conclusion (Table 5). Within clade 1-7, this result is largely caused by the interior haplotype 10, which has a significantly small D_c and a significantly small D_n. The sample size for this haplotype was large (53), and it was much more common in the east and southern parts of the study area. The explanation for the significant geographical relationship for clade 2-1 is also restricted gene flow with isolation by distance. This is because the tip clade (1-5) has significantly small D_c and D_n-values, while the interior clade has significantly large D_c and D_n-values.

The total cladogram was also significant and further supported the conclusions of restricted gene flow with isolation by distance. Using the rules provided by Posada *et al*. (2000) we concluded that 2-2 was the interior clade and 2-1 was the tip clade. This was based on the fact that clade 2-2 consisted of more haplotypes, was more common and was more widespread. Again, restricted gene flow with isolation by distance was the conclusion reached from the inference key, because the D_c and D_n-values for the tip clade were significantly small and the D_c and D_n for the interior clade were significantly large.

Table 5 Results of nested clade analysis showing clade (D_c), nested (D_n) and interior-tip clade (I-T) distances. Only clades with significant permutational chi-squared probabilities for geographical structure have been included in table. Significantly small or large values for 'D_c', D_n' and 'I-T' (D_c and D_n) are bolded and indicated by 'S' and 'L', respectively. 'Inference key ⇒ conclusion' refers to Templetons key (2000), whereby numbers indicate the steps followed and 'RG-IBD' refers to the conclusion of restricted geneflow with isolation by distance

Nesting	Clade no.	Location	D_c	D_n	Inference key ⇒ conclusion
1-Step clades					
1-7	2	Tip	2.974	3.636	1 → 2 → 11 → 17 → 4
	3	Tip	4.071	**4.057**^{L}	⇒ RG-IBD
	11	Tip	0	4.441	
	12	Tip	0	3.361	
	21	Tip	0	3.464	
	10	Interior	**3.158**^{S}	**3.452**^{S}	
	1	Interior	3.963	**3.864**^{L}	
		I-T	0.312	−0.212	
2-step clades					
2-1	1-5	Tip	**0.405**^{S}	**1.591**^{S}	1 → 2 → 3 → 4
	1-6	Interior	**2.614**^{L}	**2.402**^{L}	⇒ RG-IBD
		I-T	**2.209**^{L}	**0.811**^{L}	
Total cladogram					
	2-1	Tip	**2.234**^{S}	**3.313**^{S}	1 → 2 → 3 → 4
	2-2	Interior	**3.916**^{L}	**3.842**^{L}	⇒ RG-IBD
		I-T	**1.682**^{L}	**0.529**^{L}	

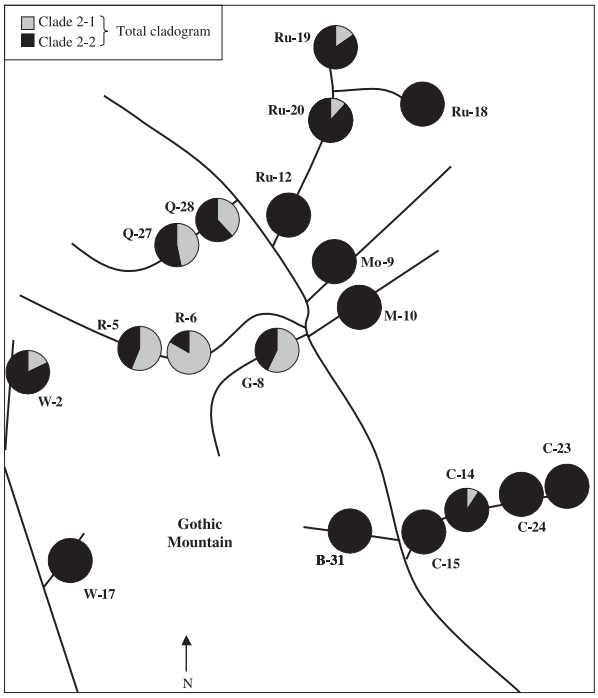

Fig. 3 A stylised map of the sampling area, showing the distribution of clades at the two-step level, identified in Fig. 2.

These results are quite surprising because they suggest not only that contemporary gene flow is restricted among streams in the study area, but also that it has been restricted historically. This is illustrated in a stylised map in Fig. 3, which shows the distribution of the two two-step clades. Clearly, as was supported by the statistical analysis, clade 2-1 has a distribution restricted mostly to creeks flowing into the west wide of the East River. Clade 2-2 seems to be more widespread, but is most common in the south and east.

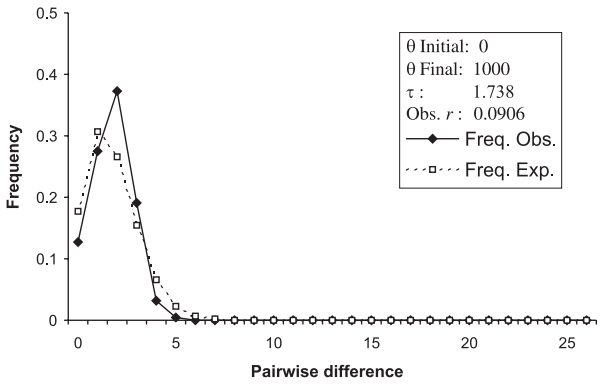

Fig. 4 Mismatch distribution based on 239 sequences of a 520 bp fragment of COI, with 19 segregating sites.

Finally, the mismatch distribution (Fig. 4) does not differ significantly from that expected following a population expansion. Based on a mutation rate of 2.3% per million years and a generation time of 12 months, this analysis suggest that these mayfly populations expanded around 150 000 years ago.

Discussion

Long-term ecological studies on this species suggest (1) *B. bicaudatus* cohorts develop relatively synchronously in a particular reach of stream (Peckarsky *et al.*, 2000) and so samples of larvae taken from a given reach are likely to be representative of the total population. (2) *Baetis* larvae are considered to be highly mobile compared with other species of stream insects (Peckarsky, 1996) and so we thought they would mix longitudinally. (3) *Baetis* adults are very small, and potentially not very strong fliers, although not much is really known about this (Edmunds *et al.*, 1976; Harker, 1992); and so we thought gene flow across sub-drainages might be limited. Given this natural history information, we hypothesised that (i) samples would not deviate from Hardy–Weinberg expectations and (ii) samples from reaches within a stream would be more similar to each another (or as similar to one another) than samples taken from different streams. The prediction regarding concordance with Hardy–Weinberg expectations was not supported, with 21% of samples deviating from Hardy–Weinberg proportions. However, unlike in the previous studies (Hughes *et al.*, 1998, 2000), many of these deviations were at a single locus, *PepB*.

The allozyme analysis also did not indicate greater genetic differences among streams than among sites within streams. Although the F_{ST}-values in this study were markedly lower than reported for the baetid species studied in sub-tropical streams (Hughes *et al.*, 2000), significant values were observed for three of the six loci in one or more streams. On the face of it this suggests both unrepresentative oviposition within reaches and lack of mixing of larvae within the stream after hatching. This result is surprising because, not only did we expect that egg masses laid in reaches would be representative of the whole population, but levels of larval drift are known to be high in these streams, especially those not containing trout (McIntosh, Peckarsky & Taylor, 2002).

The mitochondrial DNA results, however, support our original hypothesis and show that most variation is at the largest spatial scale, among streams. Furthermore, when Copper Creek site 14, was removed from the analysis, variation within streams was non-significant and almost an order of magnitude lower than variation among streams.

These conflicting results probably reflect differences between mitochondrial and nuclear genes. One explanation is that nuclear genes have not yet reached equilibrium between gene flow and genetic drift as the area was last colonised, which would have been since the last glaciation, i.e. <10 000 years ago. Nuclear genes reach equilibrium much more slowly than mitochondrial genes because of their larger effective population size (Birky *et al.*, 1984). Thus the non-significant F_{ST}-values at the largest scale could reflect lack of time to reach equilibrium rather than total panmixia.

Alternatively the difference may reflect differences in dispersal behaviour between males and females. mtDNA patterns will reflect only female dispersal, whereas patterns of nuclear genetic variation will reflect the combined effects of male and female dispersal. If females tend to remain near the stream from which they emerge, or if they fly upstream along one drainage to oviposit (as suggested by Muller, 1982; Flecker & Allan, 1988, and Peckarsky *et al.*, 2000), then mtDNA allele frequencies will be homogenised within streams each generation, but will tend to diverge between streams. In contrast, if males tend to disperse more widely and fly between drainage basins, then nuclear gene frequencies will tend to be more homogeneous, as we observed in the allozyme patterns here. Little is known about the dispersal patterns of male mayflies; but Peckarsky *et al.* (2002) have observed them swarming substantial distances from streams, implicating the potential for long distance and possibly cross-drainage dispersal. Genetic analyses of swarming male *Baetis* would help resolve this issue.

Other studies have used contrasting patterns of nuclear and mtDNA variation to infer differences in dispersal behaviour between males and females, although mostly these studies have been with vertebrates, for example, macaques (Melnick & Hoelzer, 1992) and harbour seals (Berg, Tries & Smith, 1999). Few such studies have been carried out on insects, although social hymenopterans have received some attention (e.g. Ross *et al.*, 1999).

Interestingly, the nested clade analysis suggests that not only is contemporary female dispersal limited, but also it is likely to have been limited in the past. This is because not only are some haplotypes limited in distribution, but also clade 2-1 appears to be much more common in the streams flowing from the western side than from the eastern side of the main channel. This type of pattern would be expected if emerging females tend to fly up the stream from where they emerge, as suggested earlier. Recent criticisms of nested clade analysis suggest that random lineage sorting may confuse interpretations of data (Knowles & Maddison, 2000). However, we would argue that this is unlikely in this case, as our conclusion is of limited gene flow with isolation by distance. Random lineage sorting is more likely to result in sharing of haplotypes among sites as a result of stochastic processes. Differentiation in haplotype frequencies among sites clearly implies restricted gene flow between them.

The mismatch distribution suggests that the population has expanded significantly from a much smaller population around 150 000 years ago. Obviously, the species is unlikely to have gone through this expansion in its current distribution, as the area would have been under ice. Possibly the population expanded in size in a more southerly location and invaded the East River more recently.

The very different haplotype frequencies observed between the two nearby sites in Copper Creek are difficult to explain. There is a high frequency of a rare haplotype in Copper 14. Possibly in this instance we have sampled a large number of larvae from a particular batch of eggs. More reasonably, the differences between haplotype frequencies between Copper 14 and 15 may be due to the presence of Judd Falls, which is a significant barrier to larval dispersal. This geological barrier could restrict larval movement via drift following hatching, resulting in an accumulation of individuals with distinct haplotype frequencies.

In conclusion, we suggest that the differing patterns shown by allozyme (extremely low levels of differentiation among streams) and mtDNA analyses (showing significant differentiation among streams) are explained either by the differences in evolutionary rates between the two genetic markers or by different dispersal behaviours between males and females of this species of mayflies. We suggest that males move extensively between drainages to swarm, but females tend to move upstream within drainage basins to

oviposit. The significant F_{ST}-values among sites within streams for the allozymes were unexpected, because many females contribute to larvae within a reach (Peckarsky *et al.*, 2000), and the frequency of larval drift is high (McIntosh *et al.*, 2002). However, the values were lower than those for Australian subtropical mayflies. Taken together these results suggest that adult dispersal is extensive, but that drift distances of larval mayflies may be shorter than has been previously thought (Ode, 2002).

Acknowledgements

We would like to thank Andrea Encalada, Ben Koch and Andrew Mather for help with collecting *Baetis*.

References

Berg T.M., Tries A.W. & Smith M.J. (1999) Mitochondrial and microsatellite DNA analsyies of harbour seal population structure in the northeast Pacific Ocean. *Canadian Journal of Zoology*, **77**, 930–943.

Birky C.W.J., Fuerst P. & Maruyama T. (1984) Organelle gene diversity under migration, mutation and drift: equilibrium expectation, approach to equilibrium, effects of heteroplasmic cells and comparison to nuclear genes. *Genetics*, **121**, 613–627.

Clement M., Posada D. & Crandall, K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Crandall K. (1996) Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Molecular Biology and Evolution*, **13**, 115–131.

Edmunds G.F. Jr., Jensen S.L., & Berner L. (1976) *The Mayflies of North and Central America*. University of Minnesota Press, Minneapolis, MN.

Flecker A.S. & Allan J.D. (1988) Flight direction in some Rocky Mountain mayflies (Ephemeroptera), with observations of parasitism. *Aquatic Insects*, **10**, 33–42.

Folmer O., Black M., Hoeh W., Lutz R. & Vrejenhoek R. (1994) DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Hall T.A. (1999) BIOEDIT: a user-friendly biological sequence alignment editory and analysis program for windows95/98/NT. *Nucleic Acids Symposium Sereis*, **41**, 95–98.

Harker J.E. (1992) Swarm behaviour and mate competition in mayflies (Ephemeroptera). *Journal of Zoology London*, **228**, 571–587.

Hughes J.M., Bunn S.E., Kingston D.M. & Hurwood D.A. (1995) Genetic differentiation and dispersal among populations of *Paratya australiensis* (Atyidae) in rainforest streams in south-east Queensland, Australia. *Journal of the North American Benthological Society*, **14**, 158–173.

Hughes J.M., Bunn S.E., Hurwood D.A., Choy S. & Pearson R.G. (1996) Genetic differentiation among populations of *Caridina zebra* (Decapoda: Atyidae) in tropical rainforest streams, northern Australia. *Freshwater Biology*, **36**, 289–296.

Hughes J.M., Bunn S.E., Hurwood D.A. & Cleary C. (1998) Dispersal and recruitment of Tasiagma ciliata (Trichoptera: Tasimiidae) in rainforest streams in Queensland, Australia. *Freshwater Biology*, 41, 1–10.

Hughes J.M., Mather P.B., Sheldon A. & Allendorf F. (1999) Genetic structure of stonefly (*Yoraperla brevis*) populations: the extent of gene flow among adjacent montane streams. *Freshwater Biology*, **41**, 63–72.

Hughes J.M., Bunn S.E., Cleary C. & Hurwood D.A. (2000) A hierarchical analysis of the genetic structure of an aquatic insect *Bungona* (Baetidae: Ephemeroptera). *Heredity*, **85**, 561–570.

Hughes J.M., Hillyer M. & Bunn S.E. (2003) Small-scale patterns of genetic variation in the mayfly *Bungona narilla* (Ephemeroptera: Baetidae) in rainforest streams, south-east Queensland. *Freshwater Biology*, **48**, 709–717

Hurwood D.A. & Hughes J.M. (2001) Nested clade analysis of the freshwater shrimp, *Caridina zebra* (Decapoda: Atyidae), from northeastern Australia. *Molecular Ecology*, **10**, 113–126.

Hurwood D.A., Hughes J.M., Bunn S.E. & Cleary C. (2003) Population structure in the freshwater shrimp (*Paratya australiensis*) inferred from allozymes and mitochondrial DNA. *Heredity*, **90**, 64–70.

Kerby B.M., Bunn S.E. & Hughes J.M. (1995) Factors influencing invertebrate drift in small forest streams, south-east Queensland. *Australian Journal of Marine and Freshwater Research*, **46**, 1101–1108.

Knowles L.L. & Maddison W.P. (2000) Statistical phylogeography. *Molecular Ecology*, **11**, 2623–2635.

McIntosh A.R., Peckarsky B.L. & Taylor B.W. (2002) The influence of predatory fish on mayfly drift: extrapolating from experiments to nature. *Freshwater Biology*, **47**, 1497–1514.

Melnick D.J., & Hoelzer G.A. (1992) Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. *International Journal of Primatology*, **13**, 379–393.

Miller M.P., Blinn D.W. & Keim P. (2002) Correlations between observed patterns of genetic differentiation in populations of four aquatic insect species in the

Arizona White Mountains, USA. *Freshwater Biology*, **48**, 1660–1673.

Monaghan M.T., Spaak P., Robinson C.T. & Ward J.V. (2002) Population structure of three subalpine stream insects: influences of gene flow, demographics and habitat fragmentation. *Journal of the North American Benthological Society*, **21**, 114–131.

Muller K. (1982) The colonization cycle of freshwater insects. *Oecologia*, **52**, 202–207.

Ode P.R. (2002) *The Role of Competition in the Foraging Ecology of Two Larval Mayflies, Baetis bicaudatus Banks and Epeorus deceptivus (McDunnough), (Ephemeroptera: Baetidae and Heptageniidae)*. PhD Dissertation, Cornell University, Ithaca, NY.

Peckarsky B.L. (1996) Alternative predator avoidance syndromes in stream-dwelling mayflies. *Ecology*, **77**, 1888–1905.

Peckarsky B.L., Taylor B.W. & Caudill C.C. (2000) Hydrologic and behavioral constraints on oviposition of stream insects: implications for adult dispersal. *Oecologia*, **125**, 186–200.

Peckarsky B.L., Taylor, B.W., McIntosh, A.R., McPeek, M.A., & Lytle D.A. (2001) Variation in mayfly size at metamorphosis as a developmental response to risk of predation. *Ecology*, **82**, 740–757

Peckarsky B.L., McIntosh A.R., Caudill C.C. & Dahl J. (2002) Stabilizing selection on male body size of high altitude populations of *Baetis bicaudatus* (Ephemeroptera: Baetidae). *Behavioral Ecology and Sociobiology*, **51**, 530–537.

Posada D., Crandall K.A. & Templeton A.R. (2000) GeoDos: a program for the cladistic nested analysis of the geographic distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Rank N. (1992) A hierarchical analysis of genetic variation in the montane leaf beetle *Chrysomela aenicollis* (Coleoptera: Chrysomellidae). *Evolution*, **46**, 1097–1111.

Raymond M., and Rousset F. (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Richardson B.J., Baverstock P.R. & Adams M. (1986) *Allozyme Electrophoresis:A Handbook for Animal Systematics and Population Studies*. Academic Press, Sydney.

Rogers A.R. & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**, 552–569.

Ross J.G., Shoemaker D.D., Krieger M.J.B., DeHeer C.J. & Keller L. (1999) Assessing genetic structure with multiple classes of molecular markers: a case study involving the introduced fire ant *Solenopsis invicta*. *Molecular Biology and Evolution*, **16**, 525–543.

Rozas J. & Rozas R. (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Schmidt S.F., Hughes J.M. & Bunn S.E. (1995) Gene flow among conspecific populations of *Baetis* sp. (Ephemeroptera): adult flight and larval drift. *Journal of the North American Benthological Society*, **14**, 147–157.

Schneider S., Kueffer J., Roessli D. & Excoffier L. (1997) *ARLEQUIN: Exploratory Molecular Genetics Software, Version 1.1*. University of Geneva, Switzerland.

Singer-Sam J., Tanguay R.L. & Riggs A.D. (1989) Use of Chelex to improve PCR signal from a small number of cells. *Amplifications*, **3**, 11.

Slatkin M. (1985) Gene flow in natural populations. *Annual Review of Ecology and Systematics*, **16**, 393–430.

Sweeney B.W., Funk, D.H., & Vannote, R.L. (1987) Genetic variation in stream mayfly (Insecta: Ephemeroptera) populations in Eastern North America. *Annals of the Entomological Society*, **80**, 600–612.

Sweet D., Lorente J.A., Valenzuela A., Lorente M. & Alvarez J.C. (1996) Increasing DNA extraction yield from saliva stains with a modified Chelex method. *Forensic Science International*, **83**, 167–177.

Templeton A.R. (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton A.R., Crandall, K.A. & Sing C.F. (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III Cladogram estimation. *Genetics*, **132**, 619–633.

Templeton A.R., Routman E., & Phillips C.A. (1995). Seperating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* **140**, 767–782.

Walsh P.S., Metzger D.A. & Higuchi R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, **10**, 506–513.

Wishart M.J. & Hughes J.M. (2001) Allozyme variation between conspecific populations of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), in mountain streams of the South-Western Cape, South Africa. *Freshwater Biology*, **46**, 479–490.

Wishart M.J. & Hughes J.M. (2003) Genetic population structure of the net-winged midge *Elporia barnardi* (Diptera: Blephariceridae) in streams of the south-western Cape, South Africa: implications for dispersal. *Freshwater Biology*, **48**, 28–38.

(Manuscript accepted 15 September 2003)

Appendix I Variable sites for 24 haplotypes based on 520 bp of COI

	Position																		
	5	7	1 3	1 3	1 6	1 6	2 1	2 3	2 5	2 7	3 0	3 0	3 1	3 1	3 7	4 5	4 6	4 7	5 1
Haplotype	1	2	2	5	2	5	0	8	2	4	6	9	2	5	8	0	5	2	6
1	T	G	A	A	T	T	G	C	G	A	G	G	T	C	C	C	G	C	A
2	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
5	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
6	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.
8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
9	.	A	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.
10	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.
14	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
16	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
17	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.
18	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	T	.	.
19	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.
20	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
22	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
23	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.
24	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.