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Small-scale patterns of genetic variation in the mayfly *Bungona narilla* (Ephemeroptera: Baetidae) in rainforest streams, south-east Queensland

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SUMMARY

1. Genetic structure of the mayfly *Bungona narilla* was examined using allozymes and a section of the cytochrome oxidase I gene.
2. The study had two major aims. The first was to determine whether patterns of genetic variation in mitochondrial DNA were similar to those found previously for allozymes, i.e. that more variation was evident among pools within a single stream than among streams. The mitochondrial DNA results were similar to those reported previously for allozymes, supporting the idea that larvae within any particular pool were unrepresentative of the total population and may result from a few matings.
3. The second aim was to test the hypothesis that the variation among pools within a stream was greater after dry periods than after wet periods. This was because after wet periods, larvae would have greater opportunity for mixing because of movement among pools. This hypothesis was partly supported by the mitochondrial DNA data but not by the allozyme data, in which variation among pools was extremely low on both sampling occasions. The reasons for this difference are unclear.

Keywords: allozymes, aquatic insect, genetic structure, mitochondrial DNA

Introduction

Patterns of genetic variation have been used to infer levels of dispersal among subpopulations of freshwater, marine and terrestrial organisms (e.g. Waples, 1987; Hughes *et al*., 1995; Baker, Mather & Hughes, 2000). This technique is based on the premise that, where there is extensive dispersal among populations of a species, genetic mixing will occur resulting in homogeneity of allele frequencies (Slatkin, 1981). Where dispersal is limited, populations will diverge because of the effects of genetic drift, differential selection pressures, or both. An assumption that is made when inferring dispersal using this approach is that the populations have persisted for long enough to have reached equilibrium between gene flow (tending to homogenize gene frequencies) and genetic drift (tending to lead to differentiation). The time to reach this equilibrium depends largely on the effective population size and is faster for small populations than for large ones (Crow & Aoki, 1984).

Studies of several insect species in subtropical streams in south-east Queensland have found interesting patterns of genetic variation, with the greatest levels of genetic variation at the smallest spatial scale, between pools within a single stream (Bunn & Hughes, 1997; Hughes *et al*., 1998, 2000). Similar studies in North America have also reported this effect for some species, for example for the trichopteran, *Gumaga griseola* in Arizona (Miller, Blinn & Keim, 2002). These findings are contrary to expectations of isolation by distance. One explanation for these patterns is that larvae in a single stream pool represent the offspring of

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a small number of matings, and that movement of larvae between pools within a stream is limited. Further evidence to support this idea comes from the fact that many species show significant deviations from Hardy–Weinberg proportions (randomly across sites and loci), for example a caddis (Hughes *et al*., 1998), a water strider (Bunn & Hughes, 1997) and a baetid mayfly (Hughes *et al*., 2000) species in south-east Queensland (Hughes *et al*., 1998, 2000), and a baetid (Monaghan *et al*., 2002a) and a heptageniid mayfly in Europe (Monaghan *et al*., 2002b). Furthermore, at least for one species, a tasimiid caddis fly, there was as much genetic variation between two sampling years in the same pool as there was between pools at each time (Hughes *et al*., 1998).

Although the circumstantial evidence for limited recruitment into pools is quite strong, an alternative explanation for the observed lack of differentiation at the larger spatial scales is that long-distance dispersal is limited, but there has been insufficient time to reach gene flow-drift equilibrium. Crow & Aoki (1984) give the time for populations to go half way to equilibrium as $t_{1/2} = (\ln 2)/(2\ m + 1/N_e)$, where m is the probability of migration per generation, N_e is effective population size and $t_{1/2}$ is time in generations. Thus, when N_e is large, as in many insect populations (Page & Holmes, 1998) and m is small, the time to reach equilibrium could be extremely large. Such explanations have been put forward to explain patterns of genetic variation in another stream mayfly, *Baetis alpinus*, in Europe (Monaghan *et al*., 2002a,b).

A second challenge to the interpretation of limited recruitment and restricted in-stream dispersal was presented in a recent study of allozyme variation in *Bungona narilla* (Ephemeroptera: Baetidae), in which the pattern of largest variation at the within-stream scale occurred on only two of three sampling occasions (Hughes *et al*., 2000). This was attributed to higher than average stream flows in the third year of sampling, resulting in enhanced movement of larvae within streams.

Until now, these studies have used allozyme (Schmidt, Hughes & Bunn, 1995; Hughes *et al*., 1998, 2000; Monaghan *et al*., 2002a) or AFLP (Miller *et al*., 2002) markers, representing nuclear genetic variation. Mitochondrial DNA, because it is haploid and maternally inherited, has a fourfold smaller effective population size (N_e) than nuclear DNA (Birky, Fuerst & Maruyama, 1989). For this reason, mtDNA genes should reach gene-flow/genetic drift equilibrium faster than nuclear genes. In addition, if a limited number of matings are present in a given pool, the number of mtDNA haplotypes will indicate the minimum number of females that could have contributed.

This paper has two major aims. The first is to determine if mitochondrial DNA variation in the mayfly *Bungona narilla* shows the same pattern as allozymes, with most variation among pools within a stream and effectively none among subcatchments and catchments (Schmidt *et al*., 1995; Hughes *et al*., 2000). In addition, the number of mtDNA haplotypes will be used to set the lower limit for the number of females contributing to the population of larvae in each pool.

The second aim is to test the idea that the degree of within-stream variation is a function of preceding flow conditions, by comparing samples collected after a period of prolonged flow with those collected after 6 months of limited flow in the same streams. We use both allozyme and mtDNA variation to examine this hypothesis.

Methods

Sampling design

All samples were collected from small rainforest streams in the Conondale, Blackall and D'Aguilar Ranges in south-east Queensland (Fig. 1). These ranges form the headwaters of the D'Aguilar and Stony subcatchments of the Brisbane River and the Blackall and Booloumba subcatchments of the Mary River (Fig. 1). We sampled *Bungona* on three separate sampling occasions. The first was in July 1999, when we sampled from the four subcatchments, with two or three streams in each subcatchment. For at least one stream in each subcatchment, we sampled three pools, *c.* 500 m apart. We attempted to collect 10 individuals from each pool, but this was not always possible.

In April 2000 and again in October 2000, we sampled only two creeks in the Brisbane River catchment (Kilcoy and Branch West). Prior to the April sampling, there had been prolonged periods of rain ('wet' sample), whereas for 6 months prior to the October sampling, rainfall had been very limited ('dry' sample). Four pools (sites), *c.* 500 m apart were sampled in each stream, and labelled from '1' to '4', with '1' being the most upstream site and '4' being the

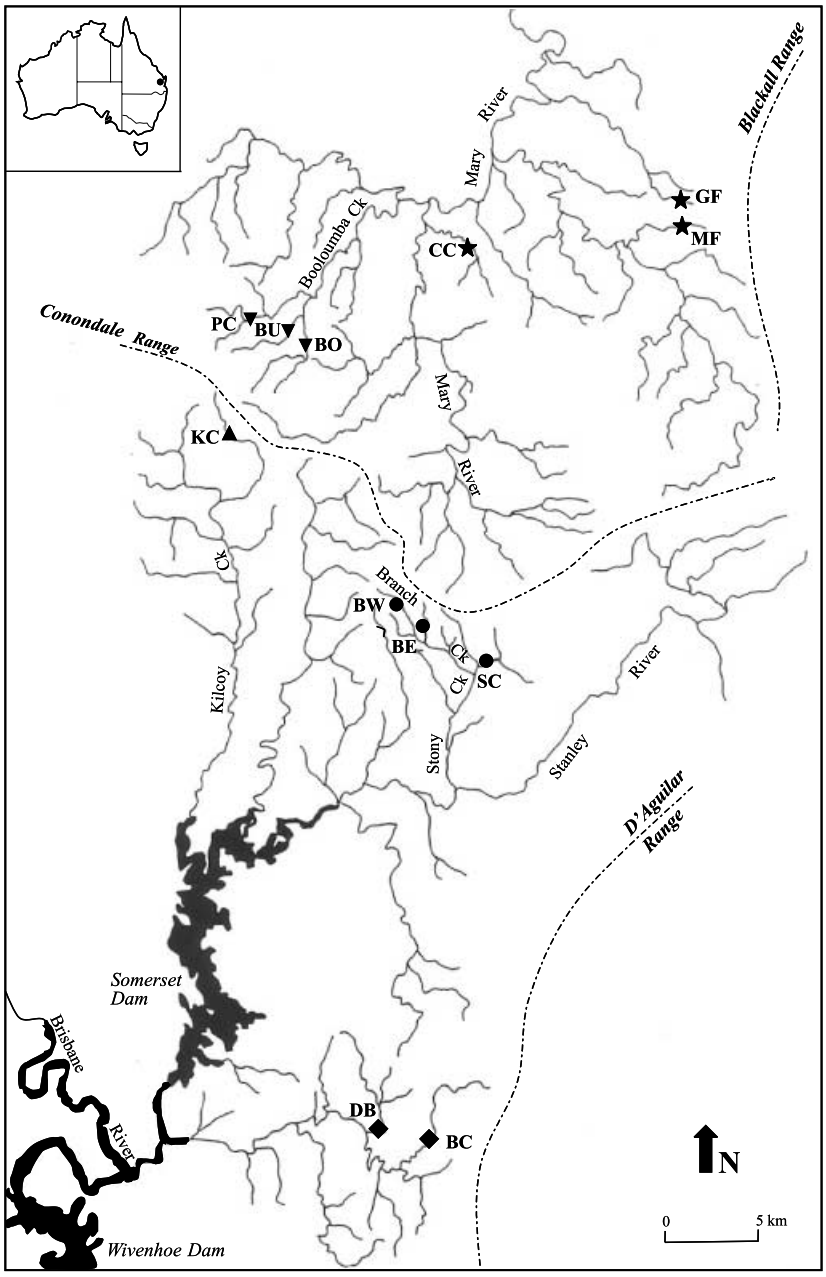

Fig. 1 Map showing sampling sites for *Bungona narilla* for the broad scale study. (◆) D'Aiguilar subcatchment (●) Stony subcatchment (▲) Kilcoy subcatchment (▼) Booloumba subcatchment (★) Blackall subcatchment. The temporal study was conducted in Kilcoy Creek and Branch West Creek.

most downstream site. We attempted to collect 50 individuals per site. On all sampling occasions, individuals were frozen in liquid nitrogen before being returned to the laboratory where they were stored at −70 °C prior to analysis.

Allozyme analysis

Samples were prepared and run on cellulose acetate plates (Helena Laboratories), using the same running and staining conditions as described in Hughes *et al*. (2000). Five enzymes were analysed: Glucose phosphate isomerase (PGI, IEC no. 5.3.1.9), Phosphoglucomutase (PGM, IEC no. 2.7.5.1), Peptidase B (PEPB, IEC no. 3.4.11) Peptidase C (PEPC, IEC no. 3.4.11) and amylase (AMY, IEC no. 3.2.1.1). Standards were run on all plates to ensure accurate scoring across sites. Standards for each plate were chosen from the previously run plate because individual samples were small.

DNA analysis

DNA was extracted from each individual using a simple chelex protocol. The head was placed in a 1.5-mL eppendorf tube containing 500 μL of 5% chelex solution and ground using a micropestle. We then added 5 μL of Proteinase K (20 mg mL^{-1}) to the samples and vortexed them before incubating them at 55 °C overnight. Prior to PCR, samples were heated at 95 °C for 10 min, then centrifuged at 15 615 *g* for 2 min at room temperature.

Two universal primers (Folmer *et al*., 1994) were used to amplify a 720-bp fragment of the cytochrome oxidase 1 gene (COI). Reactions contained 1 μL of dNTP's, 5 μL of polymerase reaction buffer, 5 μL of $MgCl_2$, 0.2 μL of *Taq* polymerase (all Promega), 2 μL of each primer (10 μM), 2 μL of DNA template, made up to a total volume of 50 μL with ddH_2O. Samples were then subjected to the following temperature profile: initial denaturation at 94 °C for 5 min, 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 stepped up to 55 °C. Finally samples were subjected to 72 °C for 5 min. PCR product was visualized and compared with a known marker on a 1.6% agarose gel using ethidium bromide.

Following sequencing of this fragment, two internal primers were designed that would amplify a smaller fragment, more appropriate for analysis using temperature gradient electrophoresis. These were BCOI_L: 5'-TGCAGAGTTGGGTAACCCTGG-3' and BCOI-H: GGCTAACACAGGTAAGGATAG-3'. Using these primers, PCR was performed using the following programme: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s.

All samples were then screened using temperature gradient gel electrophoresis with outgroup heteroduplexing (TGGE-OH) (Campbell *et al*., 1995). Prior to TGGE, 15 ng of template DNA was heteroduplexed with 15 ng of reference in a reaction containing 0.8 μL 10× ME buffer + dye, 4 μL of 8 M urea, adjusted to a total volume of 8 μL with ddH_2O. Samples were denatured at 94 °C for 5 min, heteroduplexed at 50 °C for 15 min and returned to room temperature for at least 10 min prior to electrophoresis. Optimal conditions were established by running five heteroduplexed samples (identified as different haplotypes in the initial sequencing process) on a 5% denaturing polyacrylamide gel [8 M urea, 2% glycerol, 30 : 0.5 acrylamide : bis-acrylamide in 1× ME buffer (20 mM MOPS, 1 mM EDTA)] at 300 V, for 20-min intervals from 2.5–3.8 h, with a temperature gradient of 30–50 °C. The banding patterns were visualized using silver staining (DIAGEN). The optimal conditions consisted of a gradient of 34–47 °C, with a run time of 3.1 h at 300 V.

At least one individual of each haplotype was sequenced. Prior to sequencing, purification of PCR product was performed using a QIAGEN Qiaquick PCR purification kit as per the manufacturer's instructions. Concentrations of resulting purified DNA were estimated by running 2 μL of product on a 1.6% agarose gel alongside a standard. Sequencing reactions contained 30 ng of clean DNA template, 3.2 pmole of primer, 4 μL of big dye terminator mix (Perkin Elmer) adjusted to a volume of 10 μL with ddH_2O. These were subject 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 an Applied Biosystems 377 automated sequencer.

Statistical analysis

Sequences were aligned in BioEdit (Version 5.0.9, Hall, 1999). For the broad scale study, a haplotype cladogram (network) was constructed among all sequences using TCS (Clement, Posada & Crandall, 2000). Hierarchical analysis was then performed on all three sets of data using AMOVA in Arlequin (Version 2.00, Schneider *et al*., 1997). These analyses were performed in two ways: first, using haplotype frequencies alone, and second, using haplotype frequency and sequence divergence, using number of pairwise differences. In addition to the hierarchical analysis, separate AMOVA's were performed for Kilcoy Creek and Branch Creek. This was so that results could be compared with the similar analysis performed for the allozyme data.

The allozyme data were analysed using GENEPOP (Version 3.3. Raymond & Rousset, 1995). F_{IS} values were calculated at each site for each locus separately, and deviations from Hardy–Weinberg proportions tested for significance using the exact test in GENEPOP. F_{ST} values were calculated at two hierarchical levels, among sites within each of the creeks and between the two creeks (Rank, 1992). These analyses were carried out separately for the 'wet' and 'dry' samples. F_{ST} values were tested for significance using

the formula given in Waples (1987). In addition, hierarchical analyses of genetic variation were performed in BIOSYS2 (Swofford & Selander, 1997). This analysis was used to assess the overall variation that could be attributed to variation among streams, as opposed to variation among sites within streams.

Results

Spatial patterns of variation in mtDNA

Twenty-one different haplotypes were identified, with an observed transition: transversion ratio of 9.6 : 1 and 28 variable sites of the 433 bp analysed (Table 1). Some sites (e.g. BE, BW1 and BW2) contained between one and three haplotypes, suggesting a limited number of matings. The haplotype network shows three very common haplotypes that were found across all four subcatchments and are internal within the network (Fig. 2). Interestingly, whenever a tip haplotype occurred more than once, it was found in more than one subcatchment.

The AMOVA showed no significant differentiation among subcatchments, either using haplotype frequencies alone ($F_{ST} = 0.025$, N.S.) or incorporating sequence divergence ($\Phi_{ST} = 0.014$, N.S.). When populations within subcatchments were compared, there was significant differentiation in haplotype frequencies ($F_{ST} = 0.076$, $P < 0.001$), but this differentiation became non-significant when sequence divergence data were included ($\Phi_{ST} = 0.028$, N.S.).

Dry versus wet contrasts

MtDNA data. Haplotype diversity was low at two 'dry' sites, with only two haplotypes observed in a total of 20 individuals. As in the broad scale study, this effect was not seen at all sites, with some sites having eight haplotypes of 20 individuals (Table 2).

The AMOVAS showed remarkable differences between the wet and dry samples. For the wet sample, there was no significant differentiation among sites within creeks using either method (haplotype frequency alone or sequence divergence and haplotype frequency combined) whereas both methods showed significant variation between creeks (Table 3). For the dry period, the trend was reversed, with no significant variation between creeks, but significant variation among sites within creeks. This difference was largely a reflection of significant variation within

Table 1 Table of haplotype frequencies based on CO1 sequence data for broad scale study

		Haplotype																					
Subcatchment	Site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	*n*
Blackall	GF	1	3		1			2		1										1	1		10
	MF	1	4					2	1		1												9
	CC 1		6				1	3															10
	CC 2		2					1															3
	CC 3		2				1	3	1														7
Booloumba	PC		2		1				1														4
	BO		5	1	1				1				1			1							10
	BU 1		8						1			1											10
	BU 2		3	5				1											1				10
	BU 3		7		1												1						9
Stony	BE		8																				8
	SC		1					3	1	4				1									10
	BW 1	1	6		1																		8
	BW 2		8	1	1																		10
	BW 3		6	1		1					1							1					10
D'Aguilar	DB		4				1	1	1													1	8
	BC 1		3					2	3						1				1				10
	BC 2		7					2															10
	BC 3		1						2	1				1									5

GF, Gherrula Falls; MF, Mapleton Falls; CC 1–3, Chinaman Creek sites 1–3; PC, Peter's Creek; BO, Booloumba Creek; BU 1–3, Bundaroo Creek sites 1–3; BE, Branch Creek East; SC, Stony Creek; BW 1–3, Branch Creek West sites 1–3; DB, Diana's Bath; BC 1–3, Byron Creek sites 1–3; *n*, number of individuals.

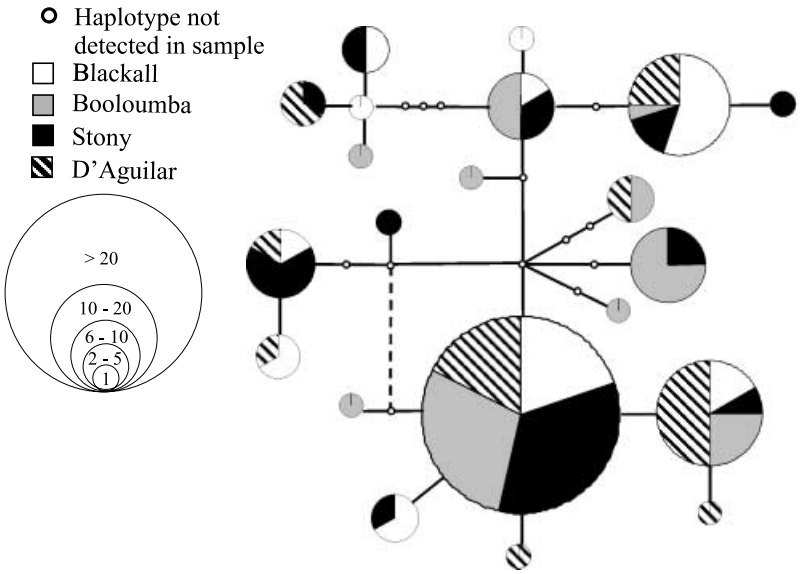

Fig. 2 Haplotype network showing relationships among COI haplotypes of *Bungona narilla* and their distribution across catchments.

Table 2 Table of haplotype frequencies based on CO1 sequence data for wet and dry samples

	Haplotype																								
Site	1	2	3	4	7	8	10	12	15	16	17	18	19	21	22	23	46	25	26	27	28	29	30	31	32	*n*
Wet																										
KC 1		6	4	3	1									1												15
KC 2		12	1	1									1	1												16
KC 3		9	2								3	2		2												18
KC 4		10	1	1				2			2			1	1											18
BW 1		13												1	1											15
BW 2		18	1											1												20
BW 3		13	1	1		1								1			1									18
BW 4		15	1						1							1										18
Dry																										
KC 1		10	3	5										1								1				20
KC 2		19		1																						20
KC 3		9	4	1						1		2			1						1				1	20
KC 4		11	2	3					1	1	1													1		20
BW 1		14		1	1								1					1	1				1			20
BW 2		16	1	2										1												20
BW 3	1	12			3		1													1						18
BW 4		19							1																	20

KC 1–4, Kilcoy Creek sites 1–4; BW 1–4, Branch Creek West sites 1–4; *n*, number of individuals.

Table 3 Results of AMOVA tests based on CO1 sequence data showing variation within and among streams for wet and dry samples

	Wet		Dry	
Hierarchical level	Φ_{ST}	F_{ST}	Φ_{ST}	F_{ST}
Among all populations	0.0775**	0.0849**	0.0526**	0.0831***
Among Kilcoy	0.0292	0.0202	0.0451*	0.0797**
Among Branch	−0.0051	−0.0166	0.0023	0.0296
Among populations within streams	0.0213	0.0089	0.0279*	0.0604**
Between streams	0.0575*	0.0768*	0.0254	0.0242

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

Table 4 Results of tests for deviations from Hardy–Weinberg (F_{IS} values given). Wet and dry samples

	Wet					Dry				
Site	*Amy-1*	*PepB-1*	*PepC-1*	*Pgi-1*	*Pgm-1*	*Amy-1*	*PepB-1*	*PepC-1*	*Pgi-1*	*Pgm-1*
KC 1	+0.333*	+0.298*	+0.472*	+0.244	+0.182	−0.008	+0.029	+0.023	−0.164	+0.009
KC 2	+0.544*	+0.254*	−0.021	+0.475*	−0.158	+0.214	+0.392*	−0.078	−0.068	+0.110
KC 3	+0.572*	+0.286*	+0.570*	+0.590*	+0.046	+0.239	−0.035	−0.039	+0.059	−0.023
KC 4	+0.080	−0.040	−0.067	−0.056	+0.138	+0.011	+0.143	+0.159	+0.159	+0.262
BW 1	−0.034	+0.193	+0.494*	−0.095	−0.015	−0.008	+0.158	−0.045	+0.165	+0.094
BW 2	+0.076	+0.325*	+0.173	−0.130	+0.290*	−0.060	+0.038	+0.325*	+0.242*	+0.238
BW 3	−0.139	−0.082	+0.169	−0.100	+0.165	−0.012	+0.207*	−0.075	+0.031	+0.051
BW 4	−0.114	+0.047	+0.374	−0.062	+0.215	+0.042	+0.058	−0.052	−0.068	+0.273

*$P < 0.05$. F_{IS} measured using Weir & Cockerham's method: +, heterozygote deficiency; –, heterozygote excess. KC 1–4, Kilcoy Creek sites 1–4; BW 1–4, Branch Creek West sites 1–4.

Table 5 F_{ST} values and levels of significance for hierarchical analysis: (1) among all populations (2) among populations in each individual creek (3) among populations within streams and (4) between streams for both wet and dry samples

	Wet						Dry					
Hierarchical level	*Amy-1*	*PepB-1*	*PepC-1*	*Pgi-1*	*Pgm-1*	All	*Amy-1*	*PepB-1*	*PepC-1*	*Pgi-1*	*Pgm-1*	All
Among all populations	0.021**	0.009	0.015	0.026***	0.006	0.016	−0.006	0.001	0.005	−0.000	0.009	0.003
Among Kilcoy	0.014	0.006	0.004	0.038***	0.012	0.018	−0.011	−0.003	0.004	0.004	0.003	−0.000
Among Branch	−0.007	0.015	0.002	−0.008	−0.007	0.001	−0.006	0.001	−0.002	−0.002	0.020*	0.006
Among populations within streams	0.014	0.012	0.009	0.020	0.004	0.011	0.000	0.004	0.004	0.003	0.013	0.006
Between streams	0.026***	0.001	0.021***	0.013***	0.004*	0.013	0.001	0.002	0.006***	−0.002	−0.002	0.001

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

Kilcoy Creek. In most cases, F_{ST} values were larger than Φ_{ST} values.

Allozyme data. Of a total of 40 tests for deviations from Hardy–Weinberg proportions, 13 were significant during the 'wet' (Table 4). There was no particular locus or site that consistently showed deviations and all showing significant deviations had positive F_{IS} values, indicating deficiencies of heterozygotes.

Following the period of high flow, only one of the F_{ST} values was significant (*Pgi-1* in Kilcoy Creek, Table 5). Four loci (*Amy-1*, *Pep-C-1*, *Pgi-1* and *Pgm-1*) showed significant F_{ST} values between the two creeks at this time.

Of 40 tests for deviations from Hardy–Weinberg proportions from the 'dry' sample (October, 2000), only four were significant. Again all were positive, indicating deficiencies of heterozygotes (Table 4). Contrary to expectations, only one locus showed a significant F_{ST} value among pools within a creek (*Pgm-1* in Branch Creek, Table 5). In addition, one locus, *PepC-1*, showed significant differentiation between the two creeks.

Discussion

As reported in earlier allozyme studies on this species (Schmidt *et al*., 1995; Hughes *et al*., 2000), differentiation in mitochondrial DNA was non-significant at the largest spatial scale. Thus there is no evidence to support a hypothesis that adult dispersal is actually limited, but that allozymes have not detected it because of insufficient time to reach gene flow-genetic drift equilibrium. Furthermore, if dispersal were limited, recently evolved haplotypes would be expected to be confined to particular subcatchments. This was not the case, because tip haplotypes that were recorded more than once always came from more than one subcatchment.

The number of haplotypes per pool varied considerably. This does not necessarily invalidate the idea that the small-scale genetic differences are due to

patchy recruitment. It is possible that some pools contain offspring from very few matings, while others are more diverse. Such a pattern might explain the differences among pools observed in previous studies (Hughes *et al*., 2000).

Interestingly, the small-scale pattern was reflected in mtDNA when haplotype frequency alone was used, but not when sequence divergence was included. This pattern would be expected because if the females ovipositing in a particular pool are a small, but random sample of the whole population, then there is no reason that their haplotypes would be particularly similar to one another (i.e. Φ_{ST} would not be significant). On the other hand, if a number of larvae have come from only two or a few females, then the frequencies of haplotypes in a pool may differ significantly from haplotype frequencies in other pools (F_{ST} would be expected to be significant).

The second aim of this study was to compare levels of genetic variation after periods of prolonged high flows with patterns observed after periods of reduced flows. The results of the allozyme and mtDNA data were somewhat contradictory. Although the difference between within-stream versus among stream allozyme differentiation was as predicted, i.e. greater after dry than after wet periods, the overall level of variation was surprisingly low after the dry period. Similarly, the number of deviations from Hardy–Weinberg proportions was actually greater after the wet than after the dry (13 of 40 comparisons after the wet and only four of 40 after the dry). Again this is contrary to predictions. Futhermore, the high levels of variation among loci that had been reported in earlier studies (Schmidt *et al*., 1995; Hughes *et al*., 2000) were less evident here, particularly for the dry sample, where all F_{ST} values were very low.

The mitochondrial DNA results partly support the expectations of the patchy recruitment hypothesis. There are significant differences among creeks, but not within creeks after prolonged wet periods, whereas there are significant differences among pools within creeks, but not between them after prolonged dry periods. However, the number of haplotypes per site did not differ between wet (4.7 per site) and dry samples (4.9 per site), as may have been expected if only a few matings were represented within pools after dry periods. It should be noted though that after the dry period, there are two sites with sample sizes of 20 individuals that contained only two haplotypes.

One explanation is that even though we attempted to sample at times where levels of rainfall had been different for the preceding few months, the length of time of low flow was insufficient for us to detect the difference because the animals we sampled for the 'dry' sample had been in the streams as very young larvae when flow rates were still high. If this were the case, then the low levels of differentiation among populations from pools within a stream would result from mixing of larvae at a very early stage. This is quite possible as the dry sample was taken after the cooler winter months, when growth rates are likely to be slower.

However, mixing of larvae before the dry period is not suggested by the mtDNA results, especially for Kilcoy Creek, which shows significant differentiation among pools after the dry period, as we had initially predicted. There is also more variation within Branch Creek after the dry period, although the values are not significant.

One possible reason for the difference between allozyme and mtDNA results could be that mtDNA is more sensitive to any lack of mixing, simply because there is more variation. For example, most allozyme loci had between two and eight alleles, whereas there were 25 mtDNA haplotypes identified in the wet/dry analysis.

Further sampling, which ensured that the entire life-cycle of sampled larvae had been completed in a wet or dry period would help to resolve these questions.

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