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Genetic diversity and historical population structure in the New Zealand mayfly *Acanthophlebia cruentata*

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SUMMARY

1. Nucleotide sequences of a 280 base pair region of the cytochrome *b* gene were used to assess genetic diversity and to infer population histories in the New Zealand mayfly *Acanthophlebia cruentata*.
2. A hierarchial examination of populations from 19 streams at different spatial scales in the central and northern North Island of New Zealand found 34 haplotypes. A common haplotype was found in all central region streams and unique haplotypes in northern streams. Several central streams had region specific haplotypes with genetically differentiated populations at the 70–100 km scale.
3. Haplotype diversity was high (0.53–0.8) at most sites, but low (0–0.22) in some central sites. AMOVA analyses found significant genetic diversity among regions (69%) and among catchments (58%). Most population pairwise F_{ST} tests were significant, with non-significant pairwise tests among sites in the central region and pairs of sites between neighbouring streams.
4. The levels of sequence divergence are interpreted as the result of Pleistocene divergence in multiple refugia, leading to the evolution of regionally unique haplotypes. The low diversity in some central region populations may result from recent colonisation following local extinctions, associated with volcanic events.

Keywords: *Acanthophlebia*, dispersal, gene flow, mtDNA cytochrome *b*

Introduction

Estimates of species dispersal potentials are important for conservation and management of aquatic resources. However for many species, especially stream insects, with two different life history stages, direct measures of dispersal are difficult and so molecular tools are being used to examine gene flow and population structure (Miller, Blinn & Keim, 2002; Monaghan *et al*., 2002; Schultheis, Weigt & Hendricks, 2002). Gene flow occurs within streams via larval drift, and within and between streams through adult flight. A hierarchical genetic analysis allows comparison of genetic differentiation within and among streams in the same and neighbouring catchments. Results from such genetic analyses have been used to infer relative dispersal patterns (Miller *et al*., 2002; Monaghan *et al*., 2002; Schultheis *et al*., 2002). Genetic studies of some North American stream insects suggest that adult flight is limited (Hughes *et al*., 1999), while for tropical stream insects in Australia, adult flight appears to be important for dispersal (Hughes *et al*., 1998). In some species marked genetic disequilibriums have been observed, possibly because of local extinctions and recolonisation (Schmidt, Hughes & Bunn, 1995; Bunn & Hughes, 1997; Monaghan *et al*., 2001).

Underlying contemporary gene flow are historical processes that have shaped present day populations, in particular repeated range expansions and contractions that occurred during the Pleistocene glacial and interglacial periods over the past two million years. Expansions and contractions sort near neutral genetic

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variation spatially and in different environmental conditions select for different alleles or haplotypes (Hewitt, 1996). Thus the application of molecular genetic markers, in addition to measuring genetic diversity, allows inferences to be made about biogeographic histories and the evolution of populations (Schultheis *et al.*, 2002). The genetic effects of Pleistocene glaciation events have been well documented for a range of terrestrial and aquatic species in continental Europe and North America (Hewitt, 1996, 2000), and increasingly for southern hemisphere freshwater fauna (Hurwood & Hughes, 2001; Waters & Wallis, 2001), although there are few reports for insects (Baker, Williams & Hughes, 2005).

In this study we used mitochondrial DNA sequence variation to measure genetic diversity in the widely distributed mayfly *Acanthophlebia cruentata* (Hudson) in the North Island of New Zealand. *Acanthophlebia cruentata* occurs in streams in native forest areas, where populations are isolated by areas of open grassland, and historically have been exposed to repeated range extensions and contractions during the Pleistocene glacial and interglacial periods. The central North Island has also been subject to extensive volcanic events over the past 40 000 years (McCraw, 1973). A previous genetic study had shown low allozyme diversity and low levels of population differentiation in *A. cruentata* when compared with the caddisfly *Orthopsyche fimbriata* (Smith & Collier, 2001). This genetic result was surprising because *A. cruentata* is thought to be a weak disperser compared with *O. fimbriata* (McLean, 1967; Collier, Wright-Stow & Smith, in review). The objective of this study was to use mtDNA markers to estimate dispersal patterns and to infer population histories of *A. cruentata*. The haploid mitochondrial genome reduces the effective size of populations, N_e, to 1/4 of that for nuclear DNA markers, making it sensitive to population bottlenecks and colonisation events (Moritz, Dowling & Brown, 1987). Limited adult dispersal will lead to genetic differentiation between catchments, with marginal populations likely to contain fewer mtDNA haplotypes.

Methods

Study organism and area

The mayfly *A. cruentata* is restricted to the North Island of New Zealand where it can be abundant in small, stony streams (Winterbourn & Gregson, 1989). *Acanthophlebia cruentata* nymphs are free-living among gravel beds in forested streams, while the adults are believed to live for only a few days and appear to stay close to the stream (Collier *et al.*, in review).

The North Island of New Zealand is a relatively narrow mountainous island with many short unconnected drainage systems. Prevailing winds flow southwest to east across the north–south lying island and much of the environment has been modified extensively for farming over the past 200 years so that areas of native forest are now highly fragmented. The North Island is north of the area where glaciers formed over the South Island during the Pleistocene, but the lowering of sea level, by up to 180 m during glacial periods, created forest-stream 'bridges' between the northern and central regions isolated by the present day isthmus, and joined Great Barrier Island to the mainland (see Fig. 1). Habitat changes, in particular extensive replacement of forest cover with open grassland and scrub in the central North Island (McGlone, Salinger & Moar, 1993) most probably led to a displacement of aquatic insects, restricted to forested streams, northwards and to low lying coastal regions. Populations were likely to have expanded outwards from these refuges during the interglacial periods, although movement would have been restricted by marine barriers created by higher sea levels. In addition volcanic events in the central North Island covered large parts of the region with pumice and ash, destroying vegetation (Leathwick & Mitchell, 1992), and presumably leading to severe perturbations or even local extinctions of aquatic populations.

Sample collection

Samples of *A. cruentata* nymphs (approximately 10 per site) were collected from 19 small gravel-bed streams, ranging in width from 2 to 8 m, in native forest catchments during November and December 2002 (Table 1). Streams were selected at different spatial scales between catchments aligned from north to south in the northern and central North Island, and within the Waikato catchment, the largest in the North Island (Fig. 1; Table 1). Northland streams (sample sites labelled N1 and N2; and WE1 and WE2) were in

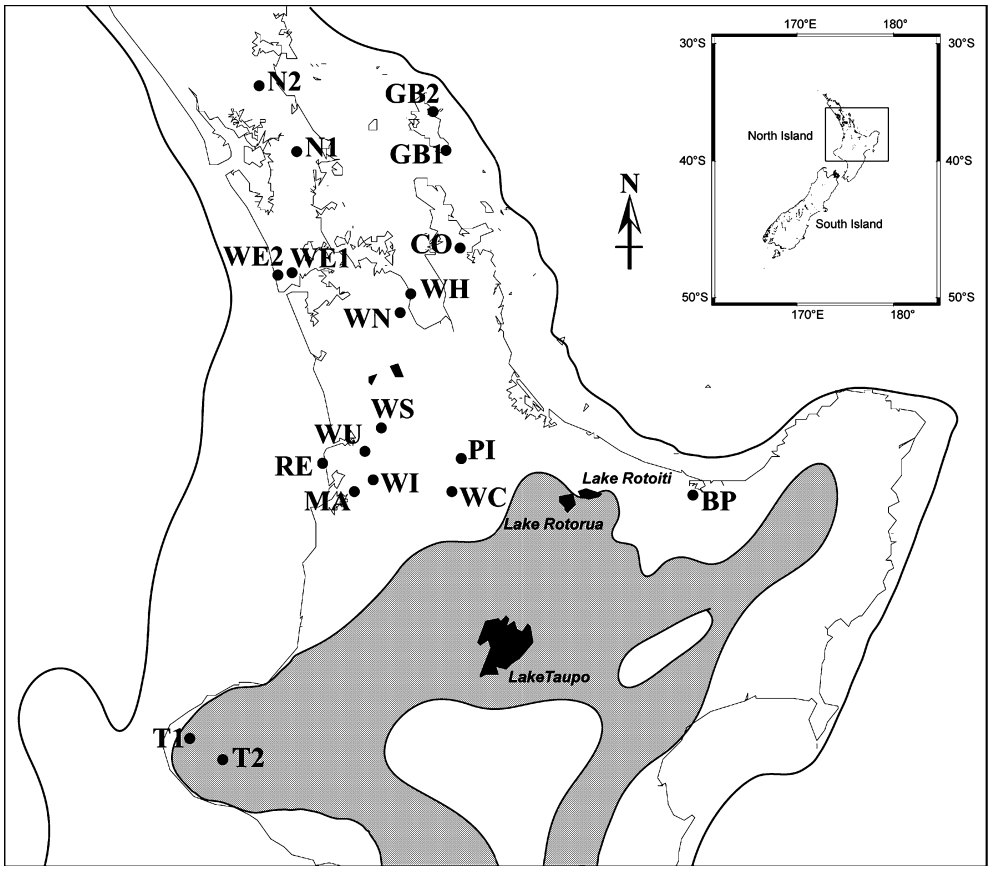

Fig. 1 Location of *Acanthophlebia cruentata* sample sites in the North Island of New Zealand. The solid line represents the coastline during the last glacial phase of the Pleistocene; the shaded area represents the area covered by open grassland and scrub during the glacial periods. Site abbreviations and location details are given in Table 1.

separate catchments. In the central North Island, streams were sampled in the Waikato River catchment (WN, WC, WI, WS), the Waitetuna catchment (WU), and separate catchments (T1, T2, RE, MA, PI, WH) from west to east across the North Island, including Coromandel (CO) and Bay of Plenty (BP) (Fig. 1). Two streams on Great Barrier Island (sites GB1 and GB2), off the east coast were included as geographic outliers. The landmass of Great Barrier Island was part of mainland New Zealand during the glacial periods (Fig. 1).

Invertebrates were collected by disturbing the streambed and collecting dislodged insects in a net held downstream. All insects were washed into a tray, and specimens of *A. cruentata* were individually frozen in dry ice or fixed in five volumes of 75% ethanol. Field identifications were based on the distinctive orange colour of *A. cruentata* (Winterbourn & Gregson, 1989; Smith, 2003).

DNA methods

Total genomic DNA was extracted by gently grinding whole nymphs in cetyltrimethyl ammonium bromide (CTAB) buffer (Reineke, Karlovsky & Zebitz, 1998) followed by digestion with proteinase-K at 55 °C for 4 h. After digestion DNA was extracted with phenol : chloroform, followed by chloroform : iso-amyl alcohol, and precipitated with 70% ethanol at −20 °C, after (Taggart *et al.*, 1992). The DNA pellet was air dried and re-suspended in 40 μL sterile water and stored at −20 °C. Part of the mitochondrial cytochrome *b* gene, was amplified using the polymerase chain reaction (PCR) in 50 μL volumes in a Cetus DNA thermocycler (Perkin-Elmer Corporation, CT, U.S.A.). The specific insect primer pair CB-J-10612; CB-N-10920 (Nucleic Acid-Protein Service Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada) that amplify an approximate

Table 1 Sample sites for *Acanthophlebia cruentata*: stream (specimen numbers in parentheses), catchment, and region

Site code	Stream	Catchment	Region
N1	Waitaraire (10)	1 Hoteo	1 Northland
N2	Kaikowhiti (10)	2 Manganui	2 Northland
WE1	Nihotupu (9)	3 Nihotupu	3 Waitakere
WE2	Marawhara (10)	4 Marawhara	3 Waitakere
GB1	Un-named (10)	5 Un-named	4 Great Barrier
GB2	Un-named (10)	6 Un-named	4 Great Barrier
WH	Whaharau (10)	7 Whaharau	5 Central, north
CO	Whangapoua (9)	8 Whangapoua	6 Coromandel
WU	Paiaka (10)	9 Waitetuna	7 Central
WN	Mangatangi (10)	10 Waikato	5 Central, north
WC	Mangatautari (9)	10 Waikato	7 Central
WS	Whatawhata (11)	10 Waipa; Waikato	7 Central
WI	Kaniwhaniwha (10)	10 Waipa; Waikato	7 Central
PI	Piakonui (9)	11 Piakonui	7 Central
RE	Te Rekereke (10)	12 Te Rekereke	8 West
MA	Mangaroa (8)	13 Mangaroa	8 West
BP	Wainui (10)	14 Wainui	9 Bay of Plenty
T1	Patea (10)	15 Patea	10 Taranaki
T2	Katikara (11)	16 Katikara	10 Taranaki

300 bp of cytochrome *b* were used with all specimens. Amplifications were carried out using an initial denaturation of 94 °C for 2 min; 34 cycles of 92 °C for 60 s, 54 °C for 60 s, and 72 °C for 90 s, followed by an extension at 72 °C for 8 min. Amplified products were separated in 1.4% agarose gels in a tris, boric acid, ethylenediaminetetra acetic acid (TBE) buffer (25 mM Tris, 0.5 mM ethylenediaminetetraacetic acid, and 25 mM boric acid), stained with ethidium bromide, and viewed under ultraviolet (UV) light. Amplifications that resulted in a single DNA product were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), and sequences were determined using the ABI Taq DyeDeoxy ™ Terminator Cycle Sequencing Kit according to the manufacturer's directions (Applied Biosystems Inc., Foster City, CA, USA). Products were sequenced in both directions using the PCR primers as sequencing primers.

Sequence alignment and data analysis

Sequences were edited in CHROMAS (Technelysium, Queensland), and aligned in the BIOEDIT programme (Hall, 1999). The cytochrome *b* sequences were aligned against the complete cytochrome *b* sequence for *Drosophila simulans* (GenBank accession nos. AF200839–45). The number of haplotypes was recorded and measures of genetic diversity were computed for each site using MEGA version 2.1 (Kumar *et al*., 2001): haplotype diversity, the probability that two haplotypes chosen at random from the sample are different, h (Nei, 1987), and nucleotide diversity, the mean number of nucleotide differences among all haplotypes in a sample, π (Nei, 1987).

Genetic differentiation

Hierarchical structure in the data was tested by partitioning variance components among and within catchments and regions with an analysis of molecular variance, AMOVA (Excoffier, Smouse & Quattro, 1992) using the ARLEQUIN (version 2.0) package (Schneider, Roessli & Excoffier, 2000). AMOVA generates an F_{ST} of standardised genetic variance (Wright, 1951) based on the frequency of haplotypes and the genetic distance between haplotypes. Permutation tests (10 000) of significance were used to test genetic structure in the data. Pairwise F_{ST}'s and exact tests of population differentiation were also computed in ARLEQUIN, and a Mantel test used to test for correlation between genetic distance (as F_{ST}) and geographic distance.

Heterogeneity in haplotype frequencies within and among regions was tested by the chi-squared randomisation test described by (Roff & Bentzen, 1989) using the REAP package (McElroy *et al*., 1992). This method overcomes the problem of a number of observed haplotypes at low frequency, by comparing chi-squared values in 1000 random rearrangements of the data. Probabilities were estimated from the number of randomisations that were equal to or greater than the observed chi-squared value. The chi-squared randomisation test was applied to pairwise comparisons of sites in the same geographical region and in the Waikato catchment to test for geographic structure. Significance levels were adjusted by the Bonferroni procedure for multiple tests after (Rice, 1989).

The existence of a molecular clock was tested with a log-likelihood ratio test (Felsenstein, 1981), using the best-fit model with and without the molecular clock restriction, and $n - 2$ degrees of freedom, where $n =$ the number of taxa. The chronology of population divergence was inferred from the net genetic divergence between clades (Avise & Walker, 1998) by

applying the widely used molecular clock divergence rate of 2% per million years, based on a divergence rate of 2.3% per million years for CO1 sequences in invertebrates (Brower, 1994), and 2.2% for insects (Gaunt & Miles, 2002).

A nested clade analysis (Templeton, 1998), was used to test for association between haplotype and geographical location. Nested clade analysis has become a valuable tool in phylogenetic analyses but is ideally combined with other statistical analyses (Stehlik, 2002), as above. A haplotype network of the 34 haplotypes, using statistical parsimony, was constructed with TCS version 1.13 (Clement, Posada & Crandall, 2000); branches were then grouped into a series of nested clades following the guidelines in Templeton (1998). GEODIS version 2.4 (Posada, Crandall & Templeton, 2000) was used to calculate clade dispersion (D_c), the average distance of individuals in clade A from the geographical center of the clade; and clade displacement (D_n), the average distance of individuals in clade A from the geographical center of the next highest nesting level. The statistics quantify differences in spatial distributions between old, or interior, clades and younger, or tip, clades in the haplotype network. A set of criteria outlined by Templeton was used to discriminate between the effects of contemporary gene flow and historical range expansions and to infer processes leading to the geographical structure (Templeton, 1998, 2004).

Results

Genetic diversity and hierarchical sub divisions within and among areas

Sequence data were collected for 280 bp of cytochrome *b* in 186 specimens. The base composition of the sequence was asymmetric and on average A, 24.0%; T, 36.3%; C, 21.2%; and G, 18.5%. Variable sites gave a transition to transversion ratio of 6.3 : 1. For the 280 bases, 248 were constant, 32 variable and parsimony uninformative, and 17 parsimony informative (Table 2). Nucleotide diversity within sites ranged from 0 in WI and WC to 0.0108 ± 0.004 in Great Barrier (Table 3).

Table 2 Variable sites for 34 haplotypes in *Acanthophlebia cruentata* cytochrome *b* sequences. Identical bases are shown by a dot. Pooled haplotypes include rare parsimony non-informative sites. Geographical site code letters as in Table 1. Haplotype positions 1–20 show the parsimony informative sites and base position.

		Position																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Haplotype	Site	19	23	25	31	49	71	79	97	124	133	163	166	169	175	190	193	208	241	247	256
1,2	N1	G	C	A	G	T	A	C	T	T	C	C	A	C	G	C	G	G	C	T	C
3	N1	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
4	N1	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
5,7	N2	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	T	.	.
6	N2	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	T	.	.
8	WN, WH	.	.	.	.	.	.	.	.	.	T	.	G	T	.	.	A	A	T	.	T
9,10,11	WN	.	.	.	.	.	.	.	.	.	T	.	G	T	A	.	A	A	T		T
12, 13	CO	.	.	.	.	.	.	.	.	C	T	.	G	T	.	.	A	A	T	.	.
14	CO	.	.	.	.	.	.	.	.	C	T	.	G	T	A	.	A	A	T	.	.
15, 34	T1, T2	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.	A	A	T	.	.
16, 30–33	COMMON	.	.	.	.	.	.	.	.	.	T	.	G	T	.	.	A	A	T	.	.
17	WE	A	.	G	.	.	.	.	.	.	T	.	.	T	.	T	A	A	T	.	.
18	WE	A	.	G	.	A	.	.	.	.	T	.	.	T	.	T	A	A	T	.	.
19	WE	A	.	G	.	A	.	.	.	.	T	.	G	T	.	T	A	A	T	.	.
20, 21	MA	.	.	.	.	.	C	.	.	.	T	.	G	T	.	.	A	A	T	.	.
22	WS, PI	.	.	.	C	.	.	.	.	.	T	.	G	T	.		A	A	T	.	.
23	GB	.	.	.	.	.	.		C	.	T	T	.	.	.	.	A	A	T	C	.
24	GB	.	.	.	.	.	.	T	C	.	T	T	.	.	.	.	A	A	T	C	.
25	GB	A	.	.	.	.	.	T	C	.	T	T	.	.	.	.	A	A	T	C	.
26	GB	A	.	.	.	.	.	.	C	.	T	T	.	.	.	.	A	A	T	C	.
27, 28	BP	.	.	.	.	.	.	.	.	.	T	.	G	T	.	T	A	A	T	.	.
29	RE	.	.	.	.	A	.	.	.	.	T	.	.	T	.	.	A	A	T	.	.

Table 3 Genetic diversity measures in *Acanthophlebia cruentata* cytochrome *b* sequences. Haplotype diversity (h) and nucleotide diversity (π); site abbreviations as in Table 1.

Site	$h \pm$ SE	$\pi \pm$ SE
WC	0.000 ± 0.00	0.0000 ± 0.000
WI	0.000 ± 0.00	0.0000 ± 0.000
WU	0.200 ± 0.15	0.0007 ± 0.001
WS	0.618 ± 0.16	0.0026 ± 0.002
PI	0.222 ± 0.17	0.0008 ± 0.001
WN	0.533 ± 0.19	0.0034 ± 0.003
WH	0.600 ± 0.13	0.0026 ± 0.002
MA	0.607 ± 0.16	0.0033 ± 0.003
RE	0.533 ± 0.09	0.0038 ± 0.003
T1	0.546 ± 0.07	0.0019 ± 0.002
T2	0.556 ± 0.09	0.0019 ± 0.002
WE1	0.694 ± 0.15	0.0051 ± 0.004
WE2	0.733 ± 0.08	0.0083 ± 0.006
GB1	0.800 ± 0.10	0.0117 ± 0.007
GB2	0.556 ± 0.16	0.0087 ± 0.006
CO	0.694 ± 0.15	0.0029 ± 0.003
BP	0.644 ± 0.10	0.0033 ± 0.003
N1	0.600 ± 0.15	0.0024 ± 0.002
N2	0.600 ± 0.13	0.0026 ± 0.002

Thirty-four haplotypes were found among the 186 specimens (Table 2). Haplotype sequences were deposited in GenBank (accession numbers AY508417–449). The numbers of haplotypes observed at each site are shown in Table 4. Several haplotypes were area specific (e.g. Waitakere, Northland, Great Barrier, Bay of Plenty, Coromandel, and Whaharau); one haplotype was common to all sites excluding the two Northland sites, while remaining haplotypes appeared in single individuals (Table 4).

Four groupings were recognised from these initial observations:

- Single sites with only unique haplotypes: Northland (sites N1 and N2);
- Single sites with unique haplotypes and a shared haplotype: Bay of Plenty (BP); Te Rekereke (RE), Mangaroa (MA), and Coromandel (CO);
- Pairs of sites with unique haplotypes and a shared haplotype: Great Barrier Island (GB1, GB2); Taranaki (T1, T2); Waitakere (WE1, WE2); central north Waikato and Whaharau (WN, WH); and
- Sites with no unique haplotypes, but shared haplotypes common to groups 2 and 3, and rare single haplotypes: the central region, Waikato (WC, WI, WS), Waitetuna (WU), and Piakonui (PI).

Haplotype diversity was 0 in WC and WI and low in WU (0.20) and PI (0.22), but otherwise ranged from 0.53 to 0.80 (Table 3). Those sites with the highest diversity, >0.69 (GB1, WE1, WE2, and CO), contained both unique regional haplotypes and the common haplotype. Genetic variation was partitioned with a nested AMOVA design by catchment and region (see catchment and region numbers in Table 1). The majority of the genetic diversity (approximately 69%) was among regions, with a non-significant diversity (1.25%) among populations within regions (including separate but neighbouring catchments), and almost 30% of genetic diversity was within populations (Table 5a). A repeat analysis based on catchments showed a similar distribution with approximately 58% diversity among catchments and approximately 31% within populations, but a significant proportion of diversity (approximately 11%) among populations within catchments (Table 5b). The within catchment diversity is largely produced by the unique haplotypes observed in the central north Waikato (WN) site within the Waikato catchment (Table 4), and is confirmed by the haplotype frequency analysis below.

Most (113 of 136) population pairwise F_{ST} tests were significant. Non-significant pairwise tests occurred among the central sites of the Waikato (WC, WI, WS), Waitetuna (WU), and Piakonui (PI) catchments; and among pairs of sites in Taranaki (T1, T2); Great Barrier Island (GB1, GB2); Waitakere (WE1, WE2); and the central north Waikato North (WN) and Whaharaua Stream (WH); and between Taranaki sites (T1, T2) and Te Rekereke (RE). Neighbouring sites with non-significant F_{ST}'s were pooled and a matrix of the pooled site comparisons showed only one pairwise comparison among the pooled sites that was non-significant, Taranaki-Te Rekereke ($F_{ST} = 0.137$). There was a significant correlation between geographic distance (measured as straight line aerial distance) and genetic distance (as pairwise F_{ST}'s) for all the mainland sites, excluding Northland ($r = 0.468$, $P = 0.006$; 1000 permutations); but not for the seven central sites ($r = 0.102$, $P = 0.344$; 1000 permutations).

Geographic structure was also tested with chi-squared randomisation tests, which showed a significant heterogeneity in haplotype frequencies among the seven sites in the central region (WU, WC, WI, WS, MA, PI, and RE). The heterogeneity in the central region was produced by the two west coast sites (MA and RE) with unique haplotypes at each site (Tables 4 and 6). There was a significant heterogeneity among

Table 4 Numbers of *Acanthophlebia cruentata* haplotypes observed at 19 sample sites. Site abbreviations as in Table 1.

Haplotype	1 N1	2 N2	3 BP	4 GB1	5 GB2	6 T1	7 T2	8 RE	9 MA	10 WN	11 WH	12 WE1	13 WE2	14 WU	15 WC	16 WI	17 CO	18 WS	19 PI
1	7																		
2	1																		
3	1																		
4	1																		
5		3																	
6		6																	
7		1																	
8										7	6								
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12																	5		
13																	1		
14																	1		
15						4	5												
16			5	3	2	5	6	6	2	1	3	1	3	9	9	10	2	7	8
17												5	4						
18												2	3						
19												1							
20									5										
21									1										
22																		1	1
23					1														
24				5	5														
25				1	1														
26				1															
27			4																
28			1																
29								4											
30														1					
31																		1	
32																		1	
33																		1	
34						1													

Source of ariation	d.f.	Sum of squares	Variance components	% of variation	*P*-value
(a) Among regions					
Among regions	9	189.65	1.147	69.2	0.000
Among populations within regions	9	6.21	0.021	1.3	0.134
Within populations	158	81.17	0.489	29.5	0.000
(b) Among catchments					
Among catchments	15	189.67	0.924	58.1	0.000
Among populations within catchments	3	6.79	0.178	11.2	0.000
Within populations	166	81.17	0.489	30.7	0.000

Table 5 Hierarchical analysis of molecular variance of mtDNA cytochrome *b* haplotypes among *Acanthophlebia cruentata* samples. (a) Among regions and (b) among catchments.

sites in the Waikato catchment (Table 6), largely produced by the northern Waikato site (WN), with haplotypes only found at this site and in a neighbouring catchment, Whaharau (WH, Table 4). Pairwise comparisons of sites found no significant differences between catchments within Taranaki, T1,

Table 6 Heterogeneity chi-squared randomisation tests for haplotype frequencies at selected *Acanthophlebia cruentata* sites in the North Island

Area (no. of sites)	χ^2-value	*P*-value
Central and west (7)	97.97	0.001*
Central excl MA and RE (5)	18.27	0.961
Central Waikato and Waitetuna (4)	14.11	0.972
Waikato (4)	43.89	<0.001*
West MA and RE (2)	11.93	0.001*
Central north WN and WH(2)	4.08	0.265
Great Barrier (2)	2.15	0.480
Waitakere (2)	2.26	0.584
Taranaki (2)	1.16	0.670

*Significant at 5% using a Bonferroni modified *P* for multiple tests.

T2; the Waitakeres, WE1, WE2; and Great Barrier Island, GB1, GB2 (Table 6); and none between neighbouring streams in the northern Waikato (WN) and Whaharau (WH) catchments; and streams in the central Waikato (WC, WI, WS), Waitetuna (WU), and Piakonui (PI) catchments. However there was significant differentiation between the Te Rekereke (RE) and Mangaroa (MA) streams, in different catchments (Table 6).

Nucleotide sequence divergence can be used to give a crude estimate of the time of phylogenetic events. Evolutionary rate variations were not detected by the likelihood ratio test for a molecular clock ($\chi^2 = 53.9$; 30, 0.005 = 53.6). Applying 2% per 10^6 years to the *Acanthophlebia* data, then the sequence divergences ranged from 0.027 between Northland and Waitakere to 0.007 between Waikato central and Waikato north, and Waikato central and Mangaroa, and equate to divergence times 0.35–1.35 Ma.

Nested clade analysis

The 34 haplotypes were fitted into thirteen 1-step clades, five 2-step clades, and two 3-step clades (Fig. 2). Haplotype 16, the common haplotype found at all sites except Northland, had the greatest outgroup probability (0.209). Nested contingency analyses revealed significant associations of clades and sampling locations: one 1-step, three 2-step clades, and both 3-step clades showed significant association with geographical location allowing rejection of the panmixia hypothesis (Table 7). The nested cladistic analysis showed significant differences for clade (D_c)

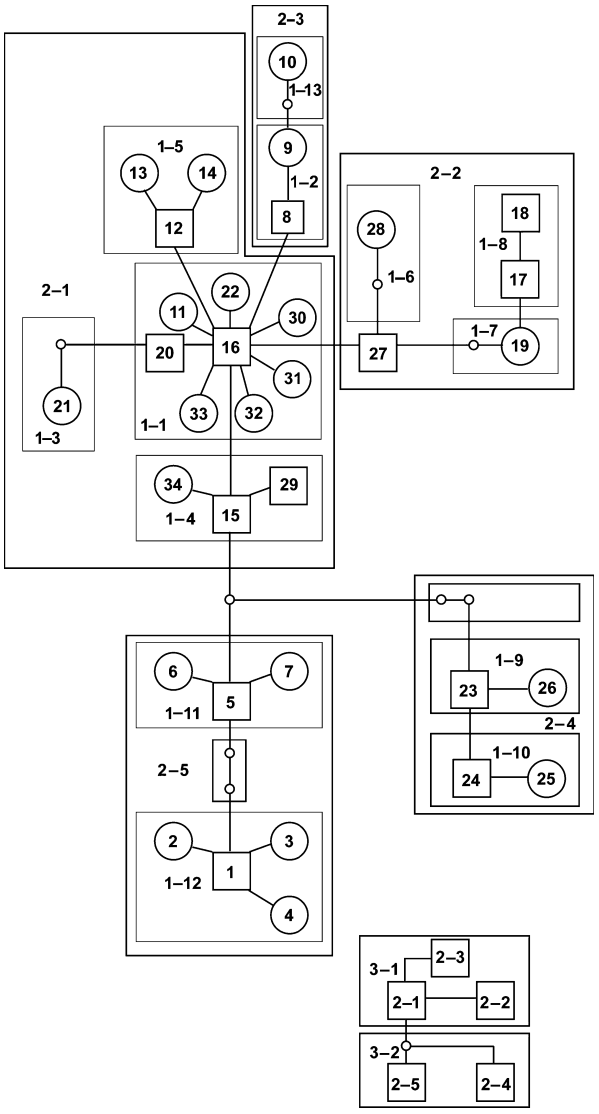

Fig. 2 Maximum parsimony network and corresponding nested design for *Acanthophlebia cruentata* cytochrome *b* haplotypes. Hypothetical haplotypes are represented by small open circles; circular haplotype boxes refer to single observations, square haplotype boxes to two or more observations. The final nesting level is shown separately.

and nested clade (D_n) distances (Table 8). Several 1-step clades were restricted to single geographical sites (1–3 = MA, 1–5 = CO, 1–11 = N1, 1–12 = N2), and some were based on a single observation (clades 1–6, 1–7, and 1–13). The stranded clade 1–6 and haplotype 27 were joined to clade 2–2 with fewer observations than clade 2–1. For most clades the widespread geographical sampling was inadequate to discriminate between competing hypotheses that lead to genetic

differentiation (Table 8). *Acanthophlebia cruentata* is abundant in forested streams throughout the North Island and our wide-scale sampling regime, designed to test regional differentiation, provided insufficient resolution to test competing genetic hypotheses given the high level of differentiation found in this first study (Tables 5 and 6). Geographically intermediate streams were not sampled between areas that displayed genetic differentiation (e.g. clade 1–4 with subclades 15 and 29 from Taranaki and Te Rekereke respectively; and clade 2–5 with sub clades 1–11 and 1–12 from sites N1 and N2 in Northland). Only clade 1–1 provided evidence for alloptaric fragmentation (Table 8), assuming that stream sampling was representative of neighbouring catchments.

Table 7 Nested contingency analysis of geographical associations for cytochrome *b* data among *Acanthophlebia cruentata* samples

Clade	χ^2-value	*P*-value
1–1	114.6	0.30
1–2	0.8	1.00
1–4	15.6	0.003
1–8	0.3	1.00
1–9	3.0	0.34
1–10	<0.01	1.00
2–1	124.7	<0.001
2–2	21.3	<0.001
2–3	0.7	1.00
2–4	0.3	1.00
2–5	20.0	<0.001
3–1	207.0	<0.001
3–2	35.0	<0.001

Discussion

Results from analyses of the cytochrome *b* sequences indicate considerable genetic differentiation among populations of *A. cruentata* in the North Island of New Zealand. Firstly there is a major break between the two northerly samples and all other sites. It is unlikely that we have sampled discrete species in these northerly sites because there are no reported morphological differences (B. Smith, NIWA, pers. comm.) and an allozyme study showed shallow genetic

Table 8 Results of the nested clade analysis of the geographical distance for cytochrome *b* haplotypes of *Acanthophlebia cruentata*

Clade	Haplotype	I/T	D_c	D_n	Chain of inference
1–1	16	I	L	L	
	20	I	S		1,2,3,4,9, No: allopatric fragmentation
1–4	15	I	S	S	
	29	T	S	L	
		I-T	S	–	1,19,20, No: inadequate geographical sampling
2–1	1–1	I	S	S	
	1–5	T	S	–	
		I-T	L	–	1,2,3,5,6,7,8, No: sampling design inadequate
2–2	1–8	T	S	S	
	1–6	T	S	L	
		I-T	L	S	1,19,20, No: inadequate geographical sampling
2–5	1–11	I	S	S	
	1–12	T	S	L	
		I-T		S	1,19,20, No: inadequate geographical sampling
3–1	2–3	T	S	–	
		I-T	L	–	1,2,3,4,9,10, No: inadequate geographical sampling
3–2	2–4	T	S	L	
	2–5	T	S	S	1,19, 20, No: inadequate geographical sampling

I/T, interior and tip clades; S/L, significantly small or large (at the 5% level) values for D_c, D_n, and I-T.
The chain of inference indicates the steps taken in the inference key to reach the conclusion (Templeton, 1998, 2004).

distances between Northland and central populations with no fixed allelic differences (Smith & Collier, 2001). Secondly regional populations, at the 70–100 km scale, are characterised by unique haplotypes; genetically differentiated populations were found in the Bay of Plenty, Coromandel, Taranaki, and northern Waikato/Whaharau. At smaller spatial scales, unique haplotypes were found in the western catchments of Mangaroa and Te Rekereke, separated by approximately 25 km. Only samples from the Waikato and neighbouring catchments in the central region, at the 20–40 km scale, showed no genetic differentiation, but were significantly different from a sample from the northern Waikato catchment approximately 70 km. The central region populations were also characterised by low haplotype diversity, with a common haplotype found in all sites.

Several studies of aquatic insects have reported significant genetic differentiation among catchments, leading the authors to conclude that adult dispersal is limited (Schultheis *et al.*, 2002; Wishart & Hughes, 2003). Finding significant genetic differentiation implies little or no contemporary gene flow, even within the same catchment. However, the converse, lack of genetic differentiation, does not necessarily imply extensive adult flight and gene flow (Monaghan *et al.*, 2001); genetic similarity between neighbouring catchments in the central region could result from historical gene flow in the absence of local selection or genetic drift and insufficient time for divergence to occur between isolated populations.

The *A. cruentata* sequence divergences indicate a mid-Pleistocene separation for the most differentiated populations, between Northland and Waitakere, based on 2% divergence per million years. There is a wide error in estimating divergence times from sequence data (Avise, 2000), and a tendency to overestimate divergence times because of the problem of ancestral polymorphisms (Edwards & Beerli, 2000). When coupled with lower substitution rates in insects (Prusser & Mossakowaski, 1998), our divergence times are likely to be under- rather than over-estimates. The Auckland isthmus has been implicated as a major barrier to dispersal for aquatic insects, based on allozyme studies of samples from Northland and the central North Island (Smith & Collier, 2001; Hogg, Willmann-Huerner & Stevens, 2002). The isthmus was covered by the Manukau Strait, between Northland and the central North Island, during the Pliocene 5–3 Ma (Stevens, 1980). The relatively shallow sequence divergences for *A. cruentata* indicate a more recent Pleistocene (<2 Ma) divergence, with the greatest divergence occurring among samples north of the Auckland isthmus. The lack of samples from the Waitakeres in the allozyme studies may have lead to an over emphasis of the importance of the Auckland isthmus in shaping genetic diversity in these species.

Acanthophlebia cruentata is restricted to small gravel streams in native forest catchments in the North Island. Assuming that it occupied similar habitats over evolutionary time, then its distribution would have expanded and contracted during the glacial and interglacial periods that dominated the central North Island during the Pleistocene. The repeated glacial and interglacial periods lead to major changes in the spread of forest cover. During the warm interglacial periods forest cover extended out from glacial refuges to cover much of the North Island, with cool temperate forest in the central North Island and warm temperate forest in Northland (Stevens, 1980). Sea levels were similar to present day: Great Barrier was an offshore island, and Northland and the Waitakeres were isolated from the central North Island by a narrow isthmus. During the glacial periods sea level was lowered by up to 180 m and the area of Great Barrier Island was part of the central-Northland land mass; likewise the area of the Waitakere ranges was part of a continuous central land mass (Fig. 1). Both Great Barrier and the Waitakeres are characterised by regional specific haplotypes from which we infer that these populations were isolated for long periods of time and evolved regionally unique haplotypes. The common, widespread haplotype is likely to be older (Templeton, Routman & Phillips, 1995). Also during the glacial periods shrubland-grassland dominated the central North Island (Stevens, 1980; McGlone, 1985), including the area of the Taranaki sample sites, and the unique haplotypes found at the present day sites may have evolved in glacial coastal areas. In general the coastal sites (Bay of Plenty, Coromandel, Te Rekereke, and Mangaroa) are characterised by unique haplotypes. Despite the high level of genetic differentiation and clear regional haplotypes found in this first mtDNA study, it was not possible to infer the evolutionary process that led to population differentiation and to distinguish between fragmentation, isolation by distance, and long distance dispersal. Intensive sampling of the numerous small uncon-

nected catchments between areas of genetic differentiation will be required to infer evolutionary histories and to define geographic barriers to dispersal. Only for the Te Rekereke and Mangaroa catchments is there evidence for allopatric fragmentation.

In addition to glacial changes influencing forest cover, the area of central New Zealand has been subject to repeated faulting and volcanic events, covering the area in ash and influencing drainage patterns. The major volcanic eruptions, especially Taupo approximately 10 000–1800 years ago, and Lakes Rotoiti and Rotorua approximately 40–15 000 years ago (see Fig. 1), filled many streams with pumice, and valleys with gas and pumice laden clouds destroying vegetation (McCraw, 1973), which was likely to have lead to local extinctions of aquatic fauna. Mayflies are very sensitive to heavy metals (Hickey & Clements, 1998), which are an important component of volcanic ash.

Local extinctions, because of ash flows, followed by re-invasions from neighbouring sites may explain the presence of a common haplotype and lower diversity in the central Waikato and Waitetuna streams. We infer that the common haplotype colonised widely during the last glacial period, and re-colonised streams in the central region 'destroyed' by volcanic events. Population histories in other species indicate reduced genetic diversity in populations occupying previously glaciated areas in the arctic (Reiss, Ashworth & Schwert, 1999). In the central North Island present day lineages of short-tailed bat *Mystacina tuberculata* may reflect recent recolonisation following the major Taupo eruption 1800 years ago (Lloyd, 2003). Non-migratory fish and the freshwater crayfish *Paranephrops planifrons* are absent east of Taupo, while the sea-migratory longfin eel is present (McDowall, 1995). Thus the present day patterns of both intraspecific genetic diversity and species distributions reflect the different dispersal potentials and abilities to re-colonise new areas following destruction by the Taupo eruption 1800 years ago. Our results for *A. cruentata* are based on one region of mtDNA and it is possible that additional markers and geographic sampling would reveal finer scale patterns of differentiation.

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