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Molecular genetic markers provide no evidence for reproductive isolation among retreat building phenotypes of the net-spinning caddisfly *Macrostemum carolina*

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Abstract

Larvae of the stream-dwelling, filter-feeding caddisfly *Macrostemum carolina* construct silken catchnets within protective retreats. In the Savannah River, *M. carolina* individuals make three different retreats, each with a distinct water entrance hole: (i) at the end of a silken tube; (ii) with a ≈180° silken backstop; and (iii) flush with the top of the retreat. To resolve whether these different retreats represent alternative behavioural phenotypes within a single panmictic population or fixed phenotypes within three genetically distinct populations or species, we compared the allele frequencies at three polymorphic nuclear loci (allozyme electrophoresis for *Gpi*, *Mpi* and *Pgm*) and the mitochondrial DNA (mtDNA) haplotype frequencies among individuals displaying the three retreat morphs. We also calculated pairwise exact tests of population differentiation using the allozyme and mtDNA allele frequencies. No significant genetic differentiation was detected among caddisflies exhibiting the different retreat morphs. Therefore, these morphs apparently represent a single panmictic population in the Savannah River. Consequently, additional study is required to assess whether this retreat polymorphism is a phenotypically plastic trait under conditional control, or is mediated by alternative alleles at a Mendelian gene or genes (or a combination of the two).

Keywords: allozyme, Hydropsychidae, mitochondrial DNA, panmixia, phenotypic polymorphism, Trichoptera

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Introduction

Non sex-correlated phenotypic polymorphisms are common in many natural populations (Mayr 1963). Such polymorphisms are mediated by plastic response to environmental heterogeneity or genetic variation at loci coding for the polymorphic trait (West-Eberhard 1989). However, polymorphisms presumed to reflect intrapopulation variation have occasionally been shown to represent fixed character states between sympatric, reproductively isolated populations or species (e.g. Douglas *et al*. 1999; Wiens *et al*. 1999). Therefore, before investigating the proximate and ultimate mechanisms maintaining morphological or behavioural polymorphisms, the genetic characteristics of individuals of each phenotype should be assessed to determine whether the phenotypes belong to one or more genetically distinct groups.

Larval net-spinning caddisflies construct silken catchnets to filter organic matter from streams. *Macrostemum carolina* (Banks) (Trichoptera: Hydropsychidae), which occur throughout the south-eastern United States (Ross 1944), construct their catchnets within protective retreats (Wallace & Sherberger 1974). In coastal plain streams with shifting sand streambeds, *M. carolina* larvae primarily inhabit submerged snags (i.e. fallen trees and branches), gouging the base of their retreat out of the wood and

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covering the top of the structure with silk. In the Savannah River, *M. carolina* individuals make three different retreats, each with a distinct water entrance hole: (i) at the end of a silken tube; (ii) with a ≈180° silken backstop; and (iii) flush with the top of the retreat (Plague & McArthur 2000), although the 'tube' and 'backstop' retreats may represent a phenotypic continuum within a single behaviour (i.e. both may function as Pitot tubes, physically pulling more water through the retreat than would flow through passively, see Plague & McArthur 2000; as such, both may be adapted for low water velocity microhabitats, while 'flush' retreats may be adapted for direct water flow in high velocity microhabitats). Unfortunately, little is known about *M. carolina*'s retreat construction behaviour through its range, although Wallace & Sherberger (1974) did note that larvae in the Apalachicola River in northern Florida construct both tube and flush retreats. Our goal was to assess whether the three retreat morphs in the Savannah River represent a polymorphic behaviour within a panmictic population or fixed strategies within reproductively isolated populations. We used both nuclear and mitochondrial DNA (mtDNA) markers for this assessment.

If these retreat morphs are genetically distinct populations, then *M. carolina* may ultimately provide empirical support for a hypothetical reproductive isolating mechanism in net-spinning caddisflies (Plague 1999). In short, Thorp (1983) suggested that the evolutionary diversification of net mesh sizes from the ancestral, large-meshed species (which inhabited high water flow velocity microhabitats) (Wallace 1975; see also Schefter 1996) was mediated largely by competitive displacement of some net-spinners into lower flow microhabitats where less water, and therefore less food, passed through their nets. Smaller meshes were advantageous because they captured smaller, more abundant food items. Plague (1999) hypothesized that this microhabitat competition may have led to speciation if it occurred between conspecifics. Because the initially large meshes of the competitively inferior caddisflies would have been inefficient in low flow microhabitats, they may have matured more slowly than the competitively superior individuals, thereby resulting in temporal reproductive isolation. Because intraspecific competition is probably still prevalent in many net-spinner populations, temporal reproductive isolation may continue to be an important evolutionary force for net-spinning caddisflies. Although it is uncertain whether *M. carolina* retreat morphs partition available snag habitats based on flow velocity, genetic divergence between them may provide initial evidence for this isolating mechanism. Alternatively, if this retreat polymorphism is expressed within a single panmictic population, then these different phenotypes likely reflect distinct genotypes at a retreat gene (or genes), or behavioural plasticity in retreat design.

Materials and methods

Collections

A total of 261 *Macrostemum carolina* larvae (50 tube retreat, 102 backstop retreat and 109 flush retreat) was collected from snags in the Savannah River (GA and SC) between 12 October 1998 and 30 March 1999. No more than five individuals of each retreat morph were collected from the same snag to minimize the likelihood of sampling only a few sibling groups. All collections were made 191–254 km upstream of the river's confluence with the Atlantic Ocean. Live caddisflies were transported back to the laboratory on ice and then stored at −80 °C until analysis.

Nuclear genetic assessment

The head and prothorax of each individual were homogenized in 10 μL of crushing buffer (1 mg NADP, 10 μL 2-mercaptoethanol, 10 mL diH_2O). Allozyme electrophoresis was performed on cellulose acetate plates as described in Hebert & Beaton (1993). All gels were run at room temperature at 200 V. A total of 39 enzyme systems was assayed initially. Subsequently, 26 presumptive loci were screened for polymorphism using 36 individuals, 12 of each retreat morph. Of these, four loci were polymorphic: *esterase* (*Est-1*; EC 3.1.1.1), *glucose-6-phosphate isomerase* (*Gpi*; EC 5.3.1.9), *mannose-6-phosphate isomerase* (*Mpi*; EC 5.3.1.8) and *phosphoglucomutase* (*Pgm*; EC 5.4.2.2). However, *Est-1* was not resolved for all individuals, possibly because of a null allele, and was not analysed further. *Gpi* was electrophoresed for 30 min in 0.02 M phosphate, pH = 7.0 buffer (plates were soaked in 0.01 M citrate-phosphate, pH = 6.4 buffer). *Mpi* and *Pgm* were electrophoresed for 35 and 45 min, respectively, in 0.1 M Tris-citrate, pH = 8.2 buffer. Alleles were numbered by decreasing electrophoretic mobility.

mtDNA genetic assessment

Total genomic DNA was extracted from frozen meso- and metathoracic tissue using a modified CTAB method (Rowan & Powers 1992) and then suspended in 100 μL of TE buffer. An ≈7500 bp segment of the mtDNA genome was amplified by long PCR (Roehrdanz & Degrugillier 1998) using conserved mtDNA primers (C2-J-3696 and CB-N-10920, Simon *et al*. 1994). Each 50 μL reaction contained 1 mM $Mg(OAc)_2$, 800 μM dNTPs, 0.64 μM of each primer, 1.2 U r*Tth* XL polymerase (GeneAmp XL PCR kit, Roche Molecular Systems, Branchburg, NJ, USA), 1× XL Buffer II and 2 μL template DNA (≈25–50 ng). To ensure reaction specificity, hot-start PCR was employed, wherein the dNTPs were not added until the reaction reached 80 °C. The PCR profile was 93 °C for 1 min; 15 cycles of 93 °C for

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50 s, 62 °C for 10 min; 25 cycles of 93 °C for 50 s, 62 °C for 10 min + 15 s autoextend per cycle; 72 °C for 7 min.

We sequenced 375 bp of the amplicon from one individual, corresponding to the cytochrome oxidase III (*COIII*) gene in *Drosophila yakuba* (Clary & Wolstenholme 1985), to confirm that the amplicon was mtDNA (GenBank Accession no. AF264048). We used internal primer C3-N-5460 (Simon *et al*. 1994) for ABI Prism BigDye terminator cycle sequencing (Applied Biosystems, Inc., Foster City, CA, USA). We then electrophoresed the resulting reaction through a polyacrylamide gel on an ABI 377 automated DNA sequencer. We are confident that this amplicon is mitochondrial because: (i) the most similar sequence in the GenBank database is the honeybee mtDNA COIII (the expected probability that they are related by chance is 2×10^{-5}), (ii) the translated amino acid sequence contains no stop codons, (iii) the four most similar protein sequences in the SwissProt database are all mtDNA COIII from flies (two *Drosophila* fruitflies and two *Anopheles* mosquitoes; the expected probability that they are related by chance is $\leq 9 \times 10^{-30}$), and (iv) although portions of the mitochondrial genome can be transferred to the nuclear genome (Zhang & Hewitt 1996), the probability of amplifying ex-mitochondrial nuclear DNA is greatly reduced when amplifying large mtDNA fragments (Roehrdanz & Degrugillier 1998).

In a preliminary study, the ≈7500 bp mtDNA amplicons were digested with 21 restriction enzymes (*Aci*I, *Afl*III, *Alu*I, *Bsr*I, *Dpn*II, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*fI, *Hha*I, *Hpa*I, *Mbo*II, *Nci*I, *Nde*I, *Nsi*I, *Pst*I, *Rsa*I, *Sau*96I, *Vsp*I, *Xmn*I). Twelve of these enzymes (*Afl*III, *Dpn*II, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hpa*I, *Mbo*II, *Nci*I, *Pst*I, *Sau*96I, *Vsp*I) worked well with unpurified PCR product (i.e. in PCR buffer) and were used to digest all samples. Restriction fragments were electrophoresed on 1–2% agarose gels, stained with either ethidium bromide or SYBR Gold (Molecular Probes, Eugene, OR, USA), and visualized under UV light. Eight individuals were not included in this analysis because we were unable to either extract or amplify their DNA (i.e. $n = 253$ for the mtDNA analysis).

Data analysis

We calculated allozyme allele frequencies and mtDNA haplotype frequencies for each retreat morph. Deviations from Hardy–Weinberg equilibrium (HWE) expected genotype frequencies were measured for each allozyme locus using an exact test of HWE (Guo & Thompson 1992), as calculated by the ARLEQUIN computer program (Schneider *et al*. 1997). To assess whether *M. carolina* is structured into reproductively isolated populations in the Savannah River, we analysed the three retreat morphs independently (assuming that each is a distinct phenotype), and with the tube and backstop retreat morphs combined (assuming that these are a continuum of a single phenotype). We used two metrics to estimate the population structure of *M. carolina*. First, we compared the allele and haplotype frequencies between retreat morphs for each locus. Because *Gpi* was the only locus conforming to χ^2 test assumptions, i.e. that no cell has an expected value <1.0, and that ≤20% of cells have expected values <5.0 (Cochran 1954), we used a Monte Carlo technique (1000 iterations) to generate a χ^2 distribution of expected values using the allele and haplotype frequencies for each locus (Roff & Bentzen 1989). Second, we calculated pairwise exact tests of population differentiation (Raymond & Rousset 1995) between each retreat morph using both the combined allozyme and the mtDNA data, again utilizing the ARLEQUIN program (Schneider *et al*. 1997). This is analogous to Fisher's exact test and uses a Markov chain algorithm to calculate the probability of nondifferentiation.

Results

All allozyme loci conform to HWE ($P > 0.05$) for the caddisflies of each retreat morph (Table 1). Each morph shares the most common allele at all loci, and only rare alleles (frequency ≤ 0.02) are not shared by all (Table 1). The allele frequencies at the three loci are roughly similar for each retreat morph (*Gpi*, $P = 0.068$; *Mpi*, $P = 0.451$; *Pgm*, $P = 0.725$; Table 1). The exact population differentiation test using the allozyme loci is also nonsignificant in each pairwise comparison (tube-backstop, $P = 0.888$; tube-flush, $P = 0.166$; backstop-flush, $P = 0.504$).

We identified 12 distinct mtDNA haplotypes with the restriction enzymes used (Table 2). Seven haplotypes were unique to a single individual, and only two were common (frequency > 0.05) in any retreat morph (Table 3). A haplotype network indicates that the mtDNA haplotypes form a star pattern, all of which are ≤2 mutations from the most common haplotype (not shown). The haplotype frequencies do not differ among morphs ($P = 0.673$; Table 3), and all pairwise exact tests of population differentiation are nonsignificant (tube–backstop, $P = 0.828$; tube–flush, $P = 0.295$; backstop–flush, $P = 0.456$).

The results are essentially identical when the tube and backstop retreat morphs are combined and compared with the flush morph. Specifically, all allozyme loci in the combined morph conform to HWE expectations (Table 1). None of the allele frequencies are different between retreat morphs (*Gpi*, $P = 0.082$; *Mpi*, $P = 0.482$; *Pgm*, $P = 0.607$; Table 1), and the pairwise exact population differentiation test using the combined allozyme data is nonsignificant ($P = 0.391$). In addition, the mtDNA haplotype frequencies are not different between morphs ($P = 0.863$; Table 3), and the pairwise exact population differentiation test using the mtDNA haplotype frequencies is nonsignificant ($P = 0.486$).

Table 1 Allele frequencies of three polymorphic allozyme loci for the three *Macrostemum carolina* retreat morphs in the Savannah River. The 'Tube + Backstop' population is the tube and backstop individuals combined, and 'Overall' is all retreat designs combined. *P*-values are those for exact tests of Hardy–Weinberg equilibrium (Guo & Thompson 1992) for each retreat morph and locus

		Retreat morph				
Locus	Allele	Tube	Backstop	Flush	Tube + Backstop	Overall
Gpi	1	0.020	0.078	0.050	0.059	0.056
	2	0.880	0.838	0.908	0.852	0.875
	3	0.100	0.083	0.041	0.089	0.069
		$P = 1.000$	$P = 0.917$	$P = 0.614$	$P = 0.853$	$P = 0.586$
Mpi	1	0.020	0.005	—	0.010	0.006
	2	—	0.005	—	0.003	0.002
	3	0.270	0.230	0.275	0.243	0.257
	4	0.700	0.745	0.706	0.730	0.720
	5	0.010	0.015	0.018	0.013	0.015
		$P = 0.083$	$P = 0.441$	$P = 0.586$	$P = 0.188$	$P = 0.226$
Pgm	1	—	0.010	—	0.007	0.004
	2	0.100	0.093	0.078	0.095	0.088
	3	0.890	0.892	0.913	0.891	0.900
	4	0.010	0.005	0.009	0.007	0.008
		$P = 1.000$	$P = 0.698$	$P = 0.564$	$P = 0.565$	$P = 0.842$

Table 2 Restriction fragments that characterize each *Macrostemum carolina* mtDNA haplotype in the Savannah River

Haplotype	*Afl*III	*Eco*RI	*Hae*III	*Hha*I	*Nci*I	*Sau*96I	*Vsp*I
1	11	001	1111	00	0000	11	111110110
2	00	001	1111	00	0000	11	111110110
3	11	111	1111	00	0000	11	111110110
4	11	001	1001	00	0000	11	111110110
5	11	001	1001	11	0000	11	111110110
6	11	001	0101	00	0000	11	111110110
7	11	001	0101	00	0110	11	111110110
8	11	001	0110	00	0000	00	111110110
9	11	001	1111	11	0000	11	111110110
10	11	001	1111	00	1001	11	111110110
11	11	001	1111	00	0000	11	101110011
12	11	001	1111	00	0000	11	111011110

Sizes of fragments: *Afl*III: 1.6 kb, 5.9; *Eco*RI: 1.5, 1.7, 4.2; *Hae*III: 1.0, 1.5, 2.1, 2.9; *Hha*I: 3.7, 3.8; *Nci*I: 1.4, 2.2, 5.3, 6.1; *Sau*96I: 2.95, 4.55; *Vsp*I: 0.28, 0.3, 0.32, 0.39, 0.42, 0.47, 0.51, 0.75, 0.81. Size fragments of invariable enzymes: *Dpn*II: 0.55, 0.8, 0.9, 2.3, 2.9; *Eco*RV: 7.5; *Hpa*I: 7.5; *Mbo*II: 0.4, 0.5, 0.7, 0.8, 1.4; *Pst*I: 7.5.

Table 3 mtDNA haplotype frequencies and sample sizes for the three *Macrostemum carolina* retreat morphs in the Savannah River. The 'Tube + Backstop' population is the tube and backstop individuals combined, and 'Overall' is all retreat designs combined

	Retreat morph				
Haplotype	Tube	Backstop	Flush	Tube + Backstop	Overall
n	48	102	103	150	253
1	0.771	0.804	0.796	0.793	0.794
2	—	0.010	—	0.007	0.004
3	—	0.010	—	0.007	0.004
4	0.146	0.157	0.136	0.153	0.146
5	—	0.010	—	0.007	0.004
6	0.021	0.010	0.010	0.013	0.012
7	—	—	0.010	—	0.004
8	—	—	0.010	—	0.004
9	0.021	—	0.010	0.007	0.008
10	0.042	—	0.010	0.013	0.012
11	—	—	0.010	—	0.004
12	—	—	0.010	—	0.004

Discussion

The allele frequencies of all loci and the population structure estimates suggest that the *Macrostemum carolina* retreat morphs are not reproductively isolated in the Savannah River. Furthermore, when the entire sample of caddisflies was pooled into a single sample for analysis ($n = 261$), all three nuclear loci were consistent with Hardy–Weinberg expectations for a randomly mating population (Table 1). Therefore, *M. carolina* apparently exhibits three retreat-building behaviours within a single panmictic population. Consequently, these retreat morphs presumably are not incipient species and as such do not support Plague's (1999) proposed reproductive isolating mechanism for net-spinning caddisflies. Nonetheless, we must at least acknowledge the possibility that the retreat

morphs may be reproductively isolated but not genetically differentiated from one another. This could be due to: (i) uniform balancing selection that homogenizes allele frequencies across all loci (e.g. Buroker 1983), although this seems unlikely because of the overall concordance between the nuclear and mtDNA genomes (see Avise 1994); or (ii) relatively recent initiation of reproductive isolation such that genetic differences have not yet accumulated at neutral loci (Neigel & Avise 1986), this would be augmented by very large effective population sizes (which may be the case, see Cudney & Wallace 1980) so that genetic drift proceeds very slowly.

Multiple discrete phenotypes can be maintained within populations by one of two proximate mechanisms (or a combination of the two): allelic variation and environmentally influenced conditional control of a phenotypically plastic trait (West-Eberhard 1989). When distinct phenotypes are produced by genetic polymorphism, i.e. different alleles at the gene (or genes) controlling character expression, the alleles exhibit Mendelian inheritance and as such every individual is genetically 'programmed' for a particular phenotype. Because neutral alleles always eventually drift to fixation, genetic polymorphisms persisting over evolutionary time must be preserved by natural selection (Hartl & Clark 1997). In general, three different selective regimes can maintain more than one allele at a locus: (i) heterozygote advantage, wherein heterozygous individuals have a higher fitness than homozygotes (e.g. Keller & Ross 1998; Carrington *et al*. 1999); (ii) negative frequency-dependent selection, wherein one genotype has a higher fitness than another when its frequency is below some threshold value but a lower fitness when its frequency is above the threshold (e.g. Hori 1993; Gillespie & Oxford 1998); and (iii) spatially variable habitats, wherein different microhabitats confer the highest fitness on different genotypes (e.g. Smith 1993). Although isolating *M. carolina*'s retreat construction genes is a sizable task, evidence for genetic polymorphism could be gained by 'forcing' individuals to construct more than one retreat in their lifetime. If individuals consistently build one type of retreat, then they may be genetically constrained to build that retreat (although this is not necessarily true, see below). Alternately, if some individuals build more than one type of retreat, then these distinct phenotypes are not strictly maintained by a genetic polymorphism.

When discrete phenotypes are mediated by conditional control mechanisms (i.e. polyphenism), individuals are genetically capable of expressing every phenotype, but each 'chooses' its phenotype based on environmental cues. In this case, different environmental cues most likely cause differential hormone production and in turn differential biochemical reactions in each phenotype, thereby generating the polyphenism (Nijhout 1999). Many discrete behavioural strategies, such as mating, dispersal and foraging, are under such conditional control (see reviews by Dominey 1984 and West-Eberhard 1989). If *M. carolina* individuals are capable of constructing different retreat types when forced to build more than one retreat, then this behavioural polymorphism is likely a conditional response to environmental variation (e.g. microgeographic location differences on the snag or water flow velocity microhabitat differences, Plague & McArthur 2000). However, this behaviour may also be environmentally cued if individuals consistently construct one type of retreat. Specifically, early instar larvae may be capable of building each retreat, with their choice depending upon the environmental conditions where they initially settle. Larvae may then become developmentally differentiated, producing a distinct suite of hormones for each retreat phenotype (Nijhout 1999). Thereafter, individuals are locked into constructing one type of retreat, and if forced to make a second retreat, must actively search for a suitable microhabitat for their retreat type (e.g. Sage & Selander 1975; Eberhard 1982).

Discrete phenotypic expression can also be maintained by combined genetic and conditional control mechanisms (e.g. Roff 1986). Partial heritability of conditionally controlled traits likely results from genetic variability of either: (i) the underlying rule 'telling' an individual which phenotype to express under certain situations, such that the same phenotype is expressed by different genotypes despite different environmental conditions; or (ii) the genetic background of nonlinked and nonpleiotropic traits, such that selection on genetic background traits results in collateral selection on the conditional control mechanism (Dominey 1984). The retreat-building behaviour in *M. carolina* may be moderated by both allelic and conditional control if some individuals make different types of retreats when forced to build more than one retreat, but if each morph phenotype tends to construct the same retreat type time and again.

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