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Historical contingency and ecological determinism interact to prime speciation in sticklebacks, *Gasterosteus*

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Historical contingency and determinism are often cast as opposing paradigms under which evolutionary diversification operates. It may be, however, that both factors act together to promote evolutionary divergence, although there are few examples of such interaction in nature. We tested phylogenetic predictions of an explicit historical model of divergence (double invasions of freshwater by marine ancestors) in sympatric species of three-spined sticklebacks (*Gasterosteus aculeatus*) where determinism has been implicated as an important factor driving evolutionary novelty. Microsatellite DNA variation at six loci revealed relatively low genetic variation in freshwater populations, supporting the hypothesis that they were derived by colonization of freshwater by more diverse marine ancestors. Phylogenetic and genetic distance analyses suggested that pairs of sympatric species have evolved multiple times, further implicating determinism as a factor in speciation. Our data also supported predictions based on the hypothesis that the evolution of sympatric species was contingent upon 'double invasions' of postglacial lakes by ancestral marine sticklebacks. Sympatric sticklebacks, therefore, provide an example of adaptive radiation by determinism contingent upon historical conditions promoting unique ecological interactions, and illustrate how contingency and determinism may interact to generate geographical variation in species diversity.

Keywords: *Gasterosteus*; species pairs; speciation; microsatellites; determinism; historical contingency

1. INTRODUCTION

A fundamental goal of evolutionary biology is to understand the processes that promote evolutionary novelty. Natural selection, stochastic processes and historical contingency are recognized as the chief influences on evolutionary trajectories (Gould & Woodruff 1990; Losos *et al*. 1998). Although the potential for interaction between contingency and determinism has been postulated (Travisano *et al*. 1995), there are few examples of such interactions that can explain variation in evolutionary diversification and species diversity in nature. The lack of natural examples of such interaction is an important gap in our understanding of lineage diversification, because specific evolutionary outcomes are usually interpreted as a result of either contingency or determinism, even within the same taxon examined for different traits (Travisano *et al*. 1995). A developing model system to examine the interaction between contingency and determinism is represented by postglacial divergences in several groups of Holarctic freshwater fishes. In these fishes, divergent forms are often found coexisting in lakes, with varying degrees of genetic and ecological isolation observed between them (reviewed in Schluter 1996*a*; Taylor 1999). For instance, divergent lineages within the lake whitefish (*Coregonus clupeaformis*) have come into secondary contact postglacially in some lakes, but not others in north-eastern North America (Bernatchez & Dodson 1990) a pattern that was probably historically contingent on shifting drainage connections among watersheds and glacial refugia (see also Svärdson 1961). In addition, in some lakes evidence has accumulated that ecological speciation, as a form of determinism, has promoted divergence within the lake whitefish complex (Lu & Bernatchez 1999). In these examples, the roles of both contingency and determinism are clearly suggested as independent processes acting within specific contexts. A relatively unexplored possibility is that of an interaction between contingency and determinism which drives evolutionary change within individual systems.

Six lakes on three separate islands in the Strait of Georgia region of south-western British Columbia contain sympatric 'benthic' and 'limnetic' species of three-spined stickleback, *Gasterosteus aculeatus* (McPhail 1994, fig. 1). Studies on the genetics, mate choice, ecology and morphology of these fish indicate that they are reproductively isolated and exploit alternative trophic niches in sympatry (Schluter & McPhail 1992; McPhail 1994; Taylor & McPhail 1999). Consequently, they fulfill the principal criteria of biological species. It has been well argued that ecological speciation, divergent selection involving exploitation of alternative trophic niches owing largely to resource competition, has been a major deterministic factor driving divergence in sticklebacks (McPhail 1993; Schluter 1994, 1996*a*,*b*; Rundle *et al*. 2000). The possibility that competition drives divergence between sympatric sticklebacks coupled with mitochondrial DNA (mtDNA) evidence for a monophyletic origin for some pairs suggests that ecological determinism may have promoted sympatric speciation (Taylor *et al*. 1997; Dieckmann & Doebeli 1999; Taylor & McPhail 1999). Indeed, similar data have been used to suggest the evolution of more species-rich assemblages of African cichlid fishes by, in large part, competitive speciation in sympatry (Reinthal & Meyer 1997).

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Table 1. *Sample sizes* (n), *average* (*s.e.*) *expected heterozygosity, average* (± *s.e.*) *number of alleles and genetic distances* (D_c *and* D_{sa}) *of benthic and limnetic pairs of stickleback from marine stickleback*

(Heterozygosity and number of alleles are averaged over six microsatellite loci. The genetic distances were calculated from 100 bootstrap replicates of the allele frequency matrix and are the averages (s.e.) of those divergences from each of seven marine populations. Sample size, heterozygosity and numbers of alleles for marine and solitary populations are the means across seven and four populations, respectively. D_c is the Cavalli-Sforza & Edwards (1967) chord distance and D_{sa} is the Bowcock *et al.* (1994) shared allele distance.)

sample	*n*	heterozygosity	number of alleles	D_c	D_{sa}
Emily limnetic	30	0.594 (0.15)	6.7 (1.4)	0.075 (0.003)	0.688 (0.024)
Emily benthic	32	0.439 (0.08)	5.7 (0.08)	0.083 (0.003)	0.722 (0.022)
Enos limnetic	25	0.627 (0.09)	6.5 (1.9)	0.073 (0.002)	0.681 (0.011)
Enos benthic	53	0.465 (0.05)	4.9 (1.7)	0.087 (0.002)	0.739 (0.014)
Paxton limnetic	28	0.743 (0.11)	10.5 (2.8)	0.073 (0.004)	0.694 (0.021)
Paxton benthic	30	0.531 (0.13)	6.2 (1.6)	0.091 (0.002)	0.786 (0.015)
Priest limnetic	33	0.681 (0.08)	8.7 (2.7)	0.073 (0.004)	0.647 (0.021)
Priest benthic	40	0.575 (0.13)	7.4 (3.0)	0.080 (0.003)	0.690 (0.020)
solitary	30.5	0.495 (0.04)	4.9 (0.48)	0.068 (0.002)	0.731 (0.024)
marine	27.4	0.798 (0.09)	10.7 (3.7)	—	—

Against this backdrop of determinism, however, is the observation that sympatric species pairs of sticklebacks are apparently restricted to a tiny portion (i.e. the Strait of Georgia) of the Holarctic range of *G. aculeatus* (McPhail 1993). The lakes containing sympatric sticklebacks were glaciated during the last (Wisconsinan) glaciation and were subjected to postglacial marine submergence. Thus, the lakes only became available for colonization by marine sticklebacks about 12 000 years before present (BP) (Hagen & McPhail 1970; McPhail 1994). The extremely limited distribution of the stickleback species pairs argues that there has been some unique historical event(s) in that area which have promoted species pair evolution, and therefore that contingency also plays a role in adaptive radiation and speciation. A historical scenario invoking contingency (McPhail 1993) posits that extant sympatric pairs result from two separate invasions of coastal lakes by ancestral marine sticklebacks following glacial recession. Benthics are thought to have evolved from a first invasion, presumably when marine sticklebacks became trapped in depressions that became lakes following marine submergence, and subsequent isostatic rebound beginning about 12 000 BP. What is unique about the Strait of Georgia region is the occurrence of a second marine submergence about 1500–2000 years after the initial submergence (Mathews *et al.* 1970). This second submergence was not as extensive as the first, but probably eliminated migration barriers between the sea and the lakes that formed after the first submergence (Mathews *et al.* 1970). McPhail (1993) postulated that this latter submergence provided a second wave of marine stickleback colonists that, finding the littoral niche occupied by benthic sticklebacks resulting from the first invasion, apparently retained the ancestral (marine) trophic role—planktivory—and evolved into limnetics. Because the double invasion model posits that benthic and limnetic species pairs were independently derived from marine ancestor, it predicts a polyphyletic relationship among species. Assuming that effective population sizes of benthics and limnetics have been relatively equal since population founding, the double invasion model also predicts a closer genetic affinity between marine and limnetic sticklebacks than between benthics and marine sticklebacks, because limnetics are thought to have been more recently derived from marine sticklebacks (Schluter & McPhail 1992; McPhail 1993).

In this study, we tested the predictions of the double invasion hypothesis by assessing the levels of genetic divergence and phylogenetic relationships among benthics, limnetics, solitary freshwater, and marine populations of sticklebacks using microsatellite DNA variation. Our analyses show how ecological determinism, recently a favoured mechanism driving divergence in vertebrates and in some freshwater fishes in particular (reviewed in Schluter 1996*a*; Taylor 1999), may be promoted by unique events in the history of particular lineages.

2. MATERIAL AND METHODS

(a) *Sampling of fish*

We surveyed allelic variation at microsatellite loci in sticklebacks collected from four of the species pair lakes as well as from four allopatric, single species (solitary) freshwater populations, and from seven marine populations (figure 1). Of the lakes with species pairs, Enos (Vancouver Island) and Paxton, Priest and Emily (Texada Island) are found on separate islands within the Strait of Georgia. On Texada Island, Paxton, Priest and Emily lakes are found in distinct drainages flowing to the north coast of the island. Consequently, marine submergences are thought to have created separate opportunities for invasion of marine sticklebacks in the three drainage systems (Enos, Paxton and Priest/Emily), although Priest and Emily lakes, are part of the same drainage system separated by 1 km of stream that is potentially navigable by sticklebacks. Emily Lake is downstream of Priest Lake and is lower in elevation (23 m versus 76 m, respectively); because the second marine submergence flooded areas at 50 m above present sea level and below (McPhail 1993), it is possible that Emily Lake was completely submerged by sea water during the most recent transgression. Consequently, Priest and Emily lakes may have been part of a larger, single drainage during deglaciation.

Details regarding field sampling, sample localities and extraction of genomic DNA from 95% ethanol-stored tissues can be found in Taylor & McPhail (1999). Six microsatellite loci

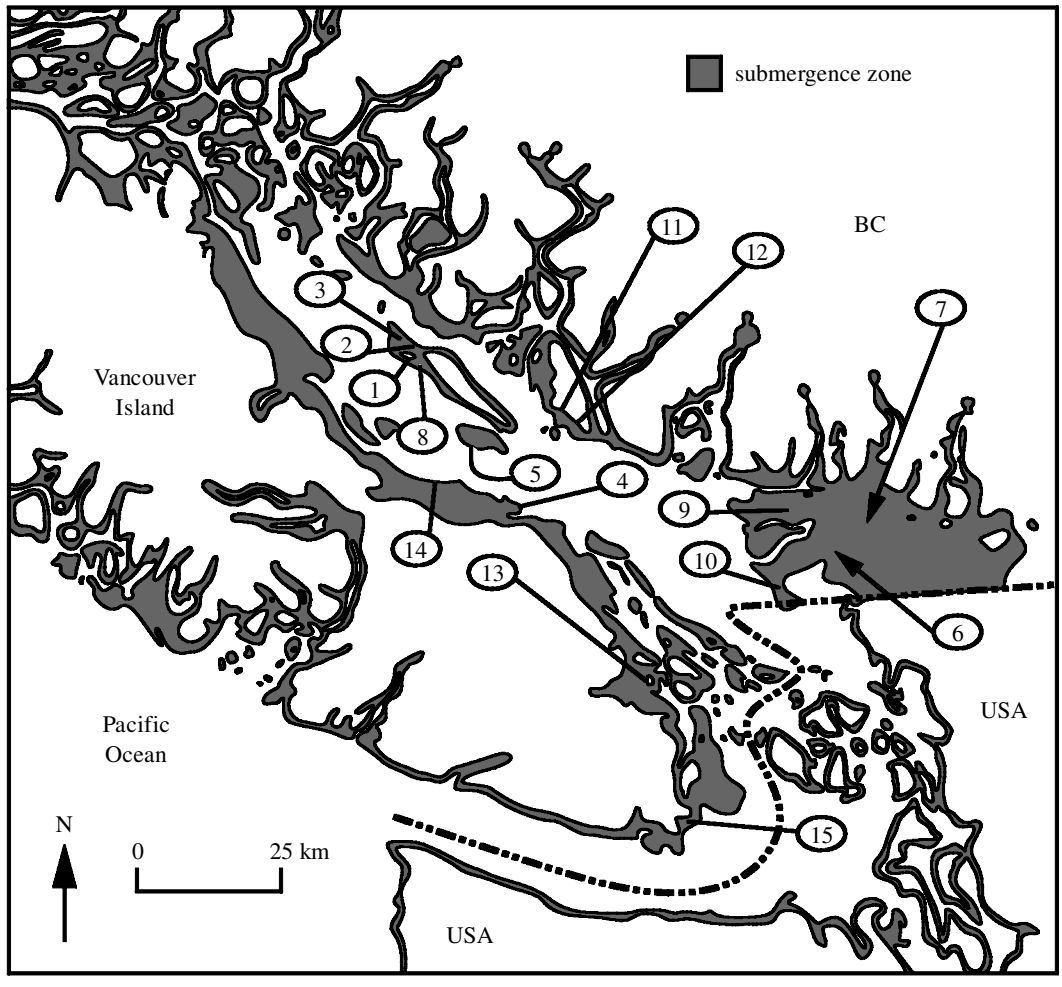

Figure 1. Locations of sticklebacks collected for microsatellite DNA analyses. Dark shading indicates maximum extent of marine submergence zone (after Mathews *et al.* 1970). 1, Paxton Lake; 2, Priest Lake; 3, Emily Lake; 4, Enos Lake; 5, Tremerton Lake; 6, Little Campbell River (freshwater); 7, Salmon River (freshwater); 8. Cranby Lake; 9, Salmon River (marine); 10, Little Campbell River (marine); 11, Salt Lagoon; 12, Oyster Lagoon; 13, Nanaimo River; 14, French Creek; 15, Witty's Lagoon.

isolated from stickleback genomic libraries were assayed using the polymerase chain reaction and radiolabelled primers as described by Rico *et al.* (1993) and Taylor (1998). Between 20 and 53 individuals were typed in each population (table 1) using procedures outlined in Taylor (1998).

(b) *Population-genetic analyses*

Populations were tested for deviations from Hardy–Weinberg equilibrium and for linkage disequilibrium between loci using GENEPOP 3.1c (Raymond & Rousset 1995). A variety of mutation- and drift-based genetic distance algorithms are available for the calculation of population subdivision and genetic distances among samples. Our results were qualitatively similar when employing a variety of models, but we considered drift-based methods of genetic distance and phylogenetic reconstruction to be the most appropriate. First, the postglacial origin of the freshwater populations of sticklebacks sets their maximum age at about 12 000 years (Mathews *et al.* 1970; McPhail 1993). Over such short time-frames, particularly when population histories may have involved large changes in population sizes, demographic processes probably overwhelm any postcolonization mutation-based differentiation patterns. Second, over such short evolutionary time-periods, drift-based or mutation-based metrics following the infinite alleles model tend to outperform alternatives based on the stepwise mutation model (Takezaki & Nei 1996; Goldstein & Pollock 1997; Paetkau *et al.* 1997), particularly in the range of the number of samples and loci employed in our study (Gaggiotti *et al.* 1999). Third, we estimated the potential importance of mutation versus drift in organizing variation among our samples by comparing the proportion of novel alleles in freshwater populations relative to marine sticklebacks. If postglacial mutation had been important in influencing allele frequency variation, we expected to see a high number of private alleles (because they are isolated from the freshwater samples) in the marine sample owing to their larger evolutionarily effective population size. In fact, the proportion of such alleles is marginally higher (25% versus 18%, $p = 0.07$) in freshwater populations, suggesting that mutation pressure has not been of primary importance in generating allele frequency variation in postglacial populations of stickleback. Rather, the trend towards a greater number of private alleles in freshwater populations probably stems from our finite sampling and the tendency for drift to be a stronger factor in smaller, freshwater populations. Consequently, we primarily employed the Cavalli-Sforza & Edwards (1967) chord distance as a drift-based measure of genetic distance for these reasons, but also employed other drift- and mutation-based genetic distances for comparative purposes (see §3(c)). Similar approaches have been adopted in other investigations of postglacial fish population genetics (e.g. Douglas *et al.* 1998;

Table 2. *Pairwise mean* F_{ST}*-values among samples of benthic and limnetic sticklebacks.*

(Also provided for comparative purposes is the mean F_{ST} among the seven marine populations and among the four solitary populations. Means were estimated by jacknifing over six loci, and confidence intervals were estimated by bootstrap analysis across loci.)

lake	F_{ST}	95% confidence interval	p
Emily	0.336	0.117–0.564	< 0.001
Enos	0.209	0.097–0.297	< 0.001
Paxton	0.213	0.113–0.301	< 0.001
Priest	0.209	0.106–0.287	< 0.001
solitary	0.298	0.093–0.391	< 0.001
marine	0.052	0.030–0.079	< 0.001

Wenburg *et al.* 1998). To summarize the degree of differentiation among samples we calculated F_{ST} as estimated by Weir & Cockerham's (1984) θ. The significance of F_{ST} was tested by permutation analyses as implemented in 'Fstat 2.8' (Goudet 1998).

Relative similarity between benthics, limnetics and marine fish was also assessed through the use of 'assignment tests' (Paetkau *et al.* 1995). This analysis uses multilocus genotypes of individuals to assign them to known populations that have been characterized at the same loci. We used the maximum-likelihood algorithms as implemented in the program 'Geneclass' (Cornuet *et al.* (1999) available at http://www.ensam.inra.fr/URLB).

(c) *Phylogenetic relationships*

Phylogenetic trees were estimated using a maximum-likelihood algorithm CONTML in PHYLIP (Felsenstein 1993) because this algorithm best fitted the assumed model of evolution by drift in the populations sampled, maximum-likelihood regularly outperforms alternative analyses (Huelsenbeck & Rannala 1997), and it allowed statistical tests of species interrelationships using the Kishino–Hasegawa test (Kishino & Hasegawa 1989). For tests of the most likely tree versus alternative topologies, trees were constrained to simulate and assess a variety of evolutionary hypotheses (e.g. single origin for benthics and limnetics, sympatric speciation within lakes). We limited our statistical tests to topological rearrangements (relative to the most likely tree) involving the fewest changes to the most likely tree, because testing every possible tree topology would result in high type I error rates. Also, our strategy made our tests conservative, and any significant rejections of the evolutionary hypotheses tested would achieve only greater significance under more extreme topology changes. We also computed neighbour-joining trees (Saitou & Nei 1987) using a variety of genetic distances (Nei's (1978) unbiased, Cavalli-Sforza & Edwards (1967) chord distance, Reynolds *et al.* (1983) coancestry coefficient, and $\delta\mu^2$, Goldstein *et al.* 1995) to compare to the results from the maximum-likelihood analyses. All genetic distance analyses were accompanied by bootstrapping over loci ($n = 100$) to derive consensus trees. All trees were constructed by arbitrarily selecting one of the marine populations, which we assume are similar to the initial founding stickleback, as the root. We also employed an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) to conduct a non-phylogenetic based test of single versus multiple origins of the stickleback species pairs. If benthics and limnetics had arisen only once each followed by colonization to each lake, then organizing all benthics into one group and all limnetics into another group should resolve the highest percentage of molecular variation. Alternatively, if the species pairs have evolved multiple times, then the 'among lakes within species' variance component should account for the greater amount of microsatellite variation. Similarly, the sympatric speciation scenario suggests that grouping species pairs by lake should account for a greater proportion of microsatellite variation relative to that between species within lakes. By contrast, the double invasion scenario would be more compatible with a higher proportion of variance between species within lakes. The AMOVAs were conducted using both F_{ST}, based on allele frequency variation only, and R_{ST}, taking allele frequency and allele size into account using the program 'Arlequin' (Schneider *et al.* 1997). The R_{ST}-based analyses consistently resolved a lower percentage of the total variation among lakes and species (see § 3(c)), supporting our assumption that drift, rather than mutation, is the primary factor influencing microsatellite allele distributions in our sample populations (see Goodman 1998).

3. RESULTS

(a) *Variability within populations*

Three tests out of 315 resulted in significant linkage disequilibrium, but none of the tests was significant after correcting for multiple comparisons. Hardy–Weinberg equilibrium was rejected in 15 out of 126 possible tests, but only five remained significant after sequential Bonferroni adjustment, but these significant tests showed no consistent pattern either among loci or populations. Four of these significant results were found in marine populations and one in a solitary freshwater population. It is possible that these results reflect our sampling of more than one genetic population within these samples. This should have no effect on our subsequent analyses because we were not concerned with resolving marine or solitary freshwater stickleback population structure *per se*, only their level of divergence from benthic and limnetic stickleback populations. The different populations of sticklebacks showed considerable variation in the extent of microsatellite heterogeneity within populations (table 1). Freshwater populations had lower mean number of alleles and expected heterozygosity (across the six loci) relative to marine sticklebacks (table 1). Within the benthic–limnetic pairs, limnetics overall had significantly (t-test, $p = 0.012$) higher heterozygosity than benthics and higher average allele numbers, but the latter difference was not significant ($p > 0.1$).

(b) *Variability among populations*

Analyses across all six loci demonstrated significant genetic divergence between species within each of the species pair lakes (table 2) and indicate that benthics and limnetics are distinct gene pools in sympatry. Standardized $F_{ST}(\theta)$-values ranged from 0.20 to 0.33 between pairs within lakes, all of which where significantly greater than zero. This level of genetic divergence between species within lakes was comparable to that observed between isolated solitary populations, but was substantially higher than that observed among marine populations (table 2), although the latter F_{ST} was still significant ($p < 0.001$).

Table 3. *Results of analysis of molecular variance on microsatellite variation in three-spined sticklebacks*

(Values to the left of the solidus represent analysis based on allele frequency variation only, those right of the solidus represent analysis incorporating differences in allele size and frequency. The grouping by species arrangement places limnetics from all lakes in one group and benthics from all lakes in another; in the grouping by lake arrangement, sympatric species are nested within 'lake'.)

hypothesis	variation	F_{ST}/ϕ_{ST}	p
grouping by species			
benthic versus limnetic	4.4/2.5	0.04/0.02	0.07/0.18
among lakes within species	20.1/11.8	0.21/0.12	<0.001/0.001
within species	75.6/85.7	0.24/0.14	<0.001/0.001
grouping by lake			
among lakes	1.89/−6.1	0.019/−0.061	0.33/0.88
between species within lakes	21.3/18.7	0.22/0.18	<0.001/0.001
within species	76.8/87.4	0.23/0.13	<0.001/0.001

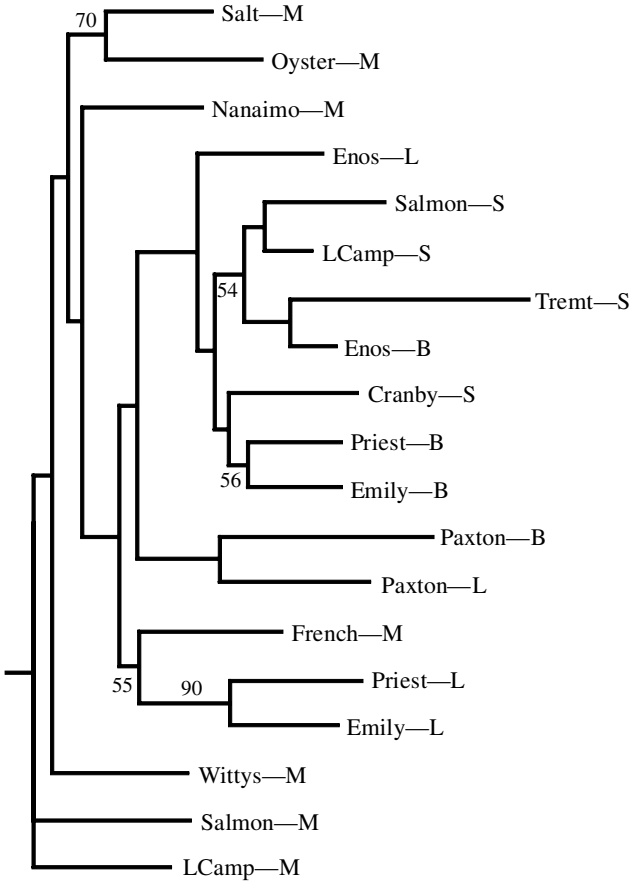

Figure 2. Maximum-likelihood tree (rooted at 'LCamp-M') of interelationships of stickleback samples. Numbers at nodes represent percentage support when >50%, from 100 bootstrap replicate analyses of the allele frequency matrix. Lake names are accompanied by an L for limnetic or B for benthic, S for solitary lake populations and M for marine populations. Other analyses produced qualitatively similar relationships with similar levels of bootstrap support.

(c) *Relationships among species*

Despite morphological and ecological similarity of benthics and of limnetics among lakes, the microsatellite data suggest that the species pairs have evolved multiple times. For instance, there was no evidence that the microsatellite variance resolved by AMOVA was structured into two major groups corresponding to 'limnetic' and 'benthic' lineages; the amount of variation attributable to species (benthic or limnetic) pooled across all lakes was low (2.5–4.4%, $p > 0.05$; table 3). By contrast, the among-lakes within-species component accounted for 10–20% of the variance and was highly significant (table 3). Furthermore, a maximum-likelihood phylogenetic analysis indicated that benthics and limnetics did not form distinct reciprocally monophyletic groups (figure 2), and this tree was significantly better than a phylogeny constrained to limnetic and benthic monophyly enforced separately (i.e. benthics monophyletic, limnetics polyphyletic and vice versa) or as reciprocally monophyletic lineages (table 4, grouping patterns 1–3). Notwithstanding our evidence for multiple origins of sympatric species pairs of sticklebacks between islands (Enos versus Paxton, Priest and Emily) and drainage systems within islands (Paxton versus Priest/Emily), our data also suggest a common origin for benthics and for limnetics in Priest and Emily lakes (figure 2). In these lakes, that are currently part of the same drainage system and separated by about 1 km of swamp and stream, the limnetics from both lakes formed one monophyletic group and the benthics from the same lakes another, suggesting that the species pairs in these lakes resulted from a single benthic–limnetic divergence.

The same general results were also observed in bootstrapped analyses of the allele frequency matrix using alternative tree-building algorithms (neighbour joining and UPGMA) based on a variety of drift-based (Cavalli-Sforza & Edwards (1967) chord distance) and mutation-based genetic distances (Nei's (1978) unbiased genetic distance, corrected $\delta\mu^2$). In these alternative analyses, again, there was no evidence of reciprocal monophyly of benthics and limnetics. As in the maximum-likelihood analysis, however, strong support was obtained for monophyly of limnetics (88–98% support) in Priest and Emily lakes. Support for monophyly of benthics in these same lakes was somewhat more modest among alternative analyses (35–64% support).

The analyses of microsatellite variation by AMOVA and maximum likelihood also argue against sympatric divergences of species within each lake. Arrangement of species pairs by lake resulted in low and non-significant variance component (<2%, $p > 0.1$, table 3). By contrast,

Table 4. *Hypotheses tested with microsatellite allele frequency variation*

(A significant result represents a rejection of the stated hypothesis. In each case, the stated hypothesis is the alternative to the pattern suggested by the most likely tree topology (figure 2).)

grouping pattern	evolutionary hypothesis	likelihood difference	s.d.	likelihood p-value
benthic monophyly	single origin	−181.9	50.8	<0.001
limnetic monophyly	single origin	−93.2	29.0	<0.001
limnetic and benthic reciprocal monophyly	single origin of both species	−116.9	30.6	<0.001
species monophyletic in each lake	sympatric speciation in each lake	−148.2	52.9	<0.01
Paxton pair not monophyletic	double invasion	−11.3	7.4	>0.05
Enos pair monophyletic	sympatric speciation	−15.7	14.8	>0.05
Priest pair monophyletic	sympatric speciation	−118.4	38.5	<0.01
Emily pair monophyletic	sympatric speciation	−112.3	51.7	<0.05
limnetics cluster closer to marine	double invasion, limnetics recent	−33.4	24.7	>0.05
benthics cluster closer to marine	double invasion, benthics recent	−254.2	40.1	<0.001

the variance component associated with differences between species within lakes was highly significant (*ca.* 20%, table 3). Consensus trees of the maximum-likelihood analyses also did not indicate majority support for monophyly of species within each lake (figure 2 and table 4, grouping pattern 4). Distinguishing between sympatric divergence and double invasion within the individual lakes was more ambiguous. For instance, Paxton Lake benthics and limnetics showed the highest support for monophyly at 44% of the maximum-likelihood trees; however, this result was not significantly better (i.e. more likely) than a tree in which this pair was not monophyletic ($p>0.05$; table 4, grouping pattern 5). Similarly, although the consensus tree did not result in monophyly of species within Enos Lake, a tree constrained to Enos Lake monophyly was not significantly worse than the consensus tree (table 4, grouping pattern 6). Only for Priest and Emily lakes was within-lake monophyly of benthics and limnetics clearly rejected (table 4, grouping patterns 7 and 8).

In addition, Paxton Lake was the only situation in which any support was obtained for monophyly of benthics and limnetics using the other genetic distances and tree construction techniques described above. Regardless of whether neighbour-joining or UPGMA was employed, Paxton limnetics and benthics were either not monophyletic ($\delta\mu^2$) or were found to be monophyletic in 38–48% of the bootstrap replicates (the genetic distances of Reynolds *et al.* (1983) and Nei (1978)). Only Cavalli-Sforza & Edwards (1967) chord distance-based analyses found majority support for monophyly of the Paxton Lake pair (57% support).

Two other aspects of the data can shed light on the likelihood of sympatric divergence within these lakes. First, when the analyses were performed on a locus-by-locus basis, of 24 possible instances of monophyly (four lake pairs × six loci), only three resulted in within-lake monophyly—Paxton Lake benthics and limnetics were monophyletic at two out of six loci and the Priest Lake species pair were monophyletic at a single locus. Further, sympatric divergence might be expected to result in many alleles that are unique to a lake and shared between co-existing species. For example, a total of 341 alleles were recorded in benthics and limnetics from the four lakes across the six loci. In only a single case, however, was there a unique (e.g. lake-specific) allele shared between benthics and limnetics (Paxton Lake, locus Gacu 7, allele 175), and this involved a total of only five fish.

The maximum-likelihood phylogenetic tree of stickleback relationships (figure 2) indicated that only in the case of Emily and Priest lakes did limnetics tend to cluster near the ancestral marine sticklebacks, while benthics from those lakes are located near the tips of the tree. Rearrangement of the consensus tree, however, with all limnetics directly descended from a node leading from all marine sticklebacks (mimicking a double invasion scenario) could not be rejected ($p>0.05$; table 4, grouping pattern 9). A similar hypothesis with benthics as most recently derived from marine sticklebacks was strongly rejected ($p<0.001$; table 4, grouping pattern 10).

Pairwise genetic distances also indicated that the limnetics in all four lakes are less divergent from marine sticklebacks than the benthics from the same lake (table 1). Solitary populations showed no consistent trend; they were the least divergent from marine sticklebacks using the chord distance measure, but most divergent using the shared allele measure (table 1). An assignment test (Paetkau *et al.* 1995; Cornuet *et al.* 1999) of individual sticklebacks based on multilocus genotypes indicated that benthics and limnetics were correctly classified with about 80% accuracy each (table 5), while solitary and marine fish were classified with a slightly greater accuracy. Within the reasonably small error rates, however, 11% of limnetics were misclassified as marine fish compared with less than 1% of benthic fish (table 5). A slightly lower percentage of limnetics were misclassified as benthics, but benthics were misclassified as solitary fish much more frequently than as limnetics and vice versa (table 5). Greater multilocus similarity between marine fish and limnetic rather than benthic (and solitary) sticklebacks was also suggested by the 6% misclassification rate of marines as limnetics compared to an error rate of 1%, and 0.5% for marine fish misclassified as benthic and solitary sticklebacks, respectively (table 5).

Table 5. *Results of jackknifed classification of individual sticklebacks from species pair (benthic and limnetic), solitary and marine populations using a Bayesian assignment procedure from variation at six microsatellite loci*

(Values shown are the number (percentage) from each source population that were classified into each potential target population.)

	classified as				
source population	limnetic	benthic	solitary	marine	total
limnetic	91 (77.9)	10 (8.2)	3 (2.9)	13 (11.0)	116
benthic	3 (1.9)	126 (81.2)	25 (16.3)	1 (0.7)	155
solitary	0 (0)	15 (12.3)	89 (73.0)	3 (2.4)	122
marine	12 (6.1)	2 (1.0)	1 (0.5)	178 (92.2)	192

4. DISCUSSION

(a) *Microsatellite variability and origin of freshwater stickleback*

Hagen & McPhail (1970) suggested that over the range of *G. aculeatus*, most freshwater populations had resulted from repeated postglacial colonizations by marine ancestral forms, and that the spectacular phenotypic diversity of freshwater sticklebacks was a result of parallel evolution. Bell (1976) suggested that this model of the evolution of freshwater sticklebacks should be reflected in reduced genetic diversity at neutral loci in recently derived freshwater populations. Our microsatellite analyses uphold this expectation because freshwater populations (benthic, limnetic, solitary) had lower allelic diversity and expected heterozygosities than did marine populations. The reduced genetic variation within founded (freshwater) populations relative to larger source (marine) populations is consistent with theoretical expectations (Nei *et al*. 1975; Hewitt 1996) and has also been observed in assays of sticklebacks using allozymes (Withler & McPhail 1985) and mtDNA (Taylor & McPhail 1999). The reduced variation in freshwater populations and knowledge of the life history of *Gasterosteus* (see § 4(c)) are consistent with the major assumption of speciation models for the evolution of stickleback species pairs, i.e. postglacial derivation of freshwater populations from marine ancestors. Differences in genetic variability between freshwater and marine sticklebacks also suggest that the demographic histories of *Gasterosteus* populations in the two environments during and following colonization of freshwater habitats have been distinct. Whether or not demographic phenomena could have played a role in the evolution of phenotypic diversity in *Gasterosteus* following colonization of freshwater (e.g. Carson & Templeton 1984) is an interesting, yet relatively unexplored, issue in these fish.

(b) *Evolution of sympatric species pairs*

Our microsatellite data show that the benthic and limnetic stickleback pairs are not distinct monophyletic lineages, and support a general mode of independent evolution of species pairs over that of common ancestry for benthic and limnetic species, respectively. These data are consistent with replicate divergences of freshwater sticklebacks inferred from morphology (Hagen & McPhail 1970), and for species pairs in particular, inferred from mtDNA (Taylor & McPhail 1999), as well as with evidence for parallel evolution in other northern fish species pairs (Pigeon *et al*. 1997; Thompson *et al*. 1997). An exception to our general result concerns the pairs in Emily and Priest lakes. In these two interconnected lakes, our data strongly suggest a common origin for benthics and limnetics, and indicate the potential importance of local dispersal in explaining the distribution of species pairs among lakes.

Our microsatellite data, however, differ from our previous mtDNA-based analysis (Taylor & McPhail 1999) in that the consensus result across the six loci suggests that benthics and limnetics within lakes are not monophyletic, but that limnetics show a more recent common ancestry with marine sticklebacks. Only the Paxton Lake pair show any hint of a monophyletic origin, but with less than majority bootstrap support (figure 2); however, when the data were examined separately over seven loci (mtDNA from Taylor & McPhail (1999), plus six nuclear DNA (nDNA)) this pair was monophyletic at only three loci (mtDNA plus two microsatellite loci). Our data, therefore, do not support the primary expectation of sympatric speciation—the within-lake monophyly of the stickleback pairs (Harrison 1991; Avise 1994)—in Paxton or Enos lakes, and monophyly of benthics and limnetics was strongly rejected in the Priest and Emily lakes' pairs (table 3).

Although analysis employing more loci may find increased support for within-lake monophyly and thus sympatric divergence, on balance, we suggest that our data are more consistent with the alternative, microallopatric 'double invasion' model of divergence (McPhail 1993). For instance, both predictions of this model, closer genetic affinity between limnetics and marine stickleback and polyphyletic origin of species with lakes, were supported by the microsatellite data. The interpretation of the genetic distance data, however, is not necessarily straightforward because limnetics also had consistently higher heterozygosity and differences in heterozygosity may contribute to differences in genetic distance (e.g. see Hedrick 1999). A possible consequence of the association between genetic distance and heterozygosity is that historical demographic differences between benthics and limnetics could confound inferences concerning recency of ancestry. For instance, if benthics have been subjected to a greater number or intensity of bottlenecks than limnetics, this could account for their greater distance from marine sticklebacks. Unfortunately, we have no direct method for evaluating this assumption historically.

Current population sizes within each lake are not well known, but a mark–recapture experiment in Enos Lake suggested that the population sizes of benthic and limnetics are roughly equal and number around 10 000 adult individuals each (B. Mathews and D. Schluter, personal communication). We also found no evidence of recent bottlenecks in any of the species pair populations using the 'mode-shift' test of Luikart *et al.* (1998) (E. B. Taylor, unpublished data). Finally, Taylor & McPhail (1999) reported higher haplotype diversity in mtDNA in benthics in three out of the four lakes (the exception was Paxton Lake where both species had very low mtDNA diversity). Given the lower effective population size of mtDNA, if, historically, lower population sizes had characterized benthics this should be reflected in consistently lower haplotype diversity in benthics, yet we observed the opposite trend in Enos, Priest and Emily lakes (Taylor & McPhail 1999). Even if there was a greater tendency for benthics to experience population bottlenecks, for which we found no evidence, this difference itself may be a consequence of the double-invasion model. If benthics did indeed evolve after the first invasion then they have had a greater time-period in small, isolated freshwater lakes that would expose them to greater potential for population fluctuations. Even with equal population sizes over time, higher levels of variation in limnetic sticklebacks is consistent with the double-invasion hypothesis because they have had fewer generations removed from the ancestral and more diverse marine populations over which heterozygosity would decay with time.

A further possibility that may explain the greater genetic similarity between limnetics and marine sticklebacks is that both forms experience a more common selective regime that favours either particular allele frequencies or greater heterozygosity in these fish. Although commonly assumed, it is unlikely that all microsatellite variation is strictly neutral (see, for example, Kashi & Soller 1999) and whenever a relatively small number of loci are examined the possibility of natural selection influencing relationships inferred from allele frequencies cannot be completely discounted. Notwithstanding the assumption of selective neutrality, and although limnetic and marine sticklebacks share a similar trophic niche (they are both planktivorous) relative to benthics, the physiological differences between residence in freshwater versus marine habitats, and the residence of limnetics and benthics within the same lake, make it unlikely that a common selective environment could explain the pattern of greater microsatellite similarity between limnetic and marine sticklebacks.

In addition to our molecular data that support the double-invasion scenario, this hypothesis is consistent with (i) the geological history of the Strait of Georgia region, (ii) the occurrence of freshwater sticklebacks largely only in areas subject to marine submergences, (iii) the presence of sympatric pairs of sticklebacks only in the central Strait of Georgia area that was subject to two marine submergences (Mathews *et al.* 1970; McPhail 1993), (iv) founder effect-induced lower levels of molecular variation in freshwater sticklebacks (table l; Withler & McPhail 1985; Taylor & McPhail 1999), (v) the present day seasonal movements of marine sticklebacks into freshwater streams for breeding (Hagen & McPhail 1970; McPhail 1994), and (vi) with the observation that most solitary populations are more similar to benthics in morphology and ecology (McPhail 1994). The greater microsatellite similarity between marine and limnetic sticklebacks is consistent with previous allozyme (Withler & McPhail 1985; McPhail 1994) and physiological (salinity tolerance) (Kassen *et al.* 1995) data showing that within each species pair the limnetics are closer to marine ancestors than are the benthics. Our results, therefore, support the double-invasion model of species pair evolution and imply a crucial role for historical contingency in adaptive radiation. The species pairs probably would not have evolved had the lakes not been subject to temporally distinct invasions by marine sticklebacks.

(c) *Gene flow and speciation in sticklebacks*

The differences between previous mtDNA and current microsatellite phylogenies for stickleback species pairs are similar to discordances between mtDNA and nuclear-based phylogenies observed in other taxa (Tegelström 1987; Arnold 1997). The apparent monophyly for some stickleback species pairs derived from mtDNA data could result from mtDNA introgression between species if reproductive isolation was not complete upon secondary contact. In fact, morphological analyses suggest that a limited degree of hybridization between benthic and limnetic species occurs in at least two lakes (Paxton and Enos) and experimental crosses indicate hybrids are completely viable and fertile under laboratory conditions (McPhail 1994; Hatfield 1997). In addition, mtDNA transfer between species is not uncommon, particularly if one species is rare (Tegelström 1987; Arnold 1997). Mitochondrial DNA is also expected to retain the signature of hybridization more faithfully than nDNA owing to the lack of recombination, its uniparental mode of inheritance, and because, in the case of sticklebacks, bottlenecks in freshwater populations could result in rapid chance fixation of introgressed mtDNA within lakes owing to its reduced effective population size (Arnold 1997; Wang *et al.* 1997). It is also possible that selection against introgression of nuclear loci may be stronger than for cytoplasmically inherited markers, or that mtDNA introgression may itself reflect positive selection (Bernatchez *et al.* 1995; Arnold 1997). These possibilities indicate that either neutrality or positive selection of mtDNA variants and hybridization could compromise phylogenetic reconstruction based only on mtDNA, and suggest that a more robust test should include multiple markers as in our current analysis. The pattern of monophyly of lake pairs at some loci (e.g. Paxton Lake with mtDNA, some microsatellites) and not others is exactly the pattern predicted to occur if the taxa being assayed have hybridized in the past (e.g. Wang *et al.* 1997).

We have presented evidence for the importance of a micro-allopatric model invoking double invasions of freshwater as a plausible mechanism for initiating the evolution of species pairs of sticklebacks. Further, despite some hybridization and mtDNA data that imply gene-flow, sympatric benthic and limnetic sticklebacks maintain themselves as distinct genomes and display strong assortative mating and clear resource partitioning. Apparently, some gene flow has occurred, but benthics

and limnetics in each lake persist as biological species, and our analyses suggest that genetic differentiation can be maintained in the face of gene flow. Persistence of genetic differentiation in the face of gene flow following the second invasion in sticklebacks, however, raises the possibility that interactions in sympatry could also have played a role in promoting divergence in *Gasterosteus* (Rice & Hostert 1993; Rundle & Schluter 1998). Microsatellites and mtDNA data (Taylor & McPhail 1999) support independent evolution of the species pairs, and these divergences have probably been driven in large part by ecological factors (McPhail 1993; Schluter 1994, 1996*a*,*b*). Speciation in three-spined sticklebacks therefore highlights the role of parallel evolution and determinism in the evolution of communities during adaptive radiations (Schluter 1994, 1996*a*,*b*). The fact that sympatric pairs of sticklebacks are endemic to the Strait of Georgia hints that this area harbours a special feature or has undergone some unique historical event to promote species pair evolution. Our microsatellite data suggest that double invasions of freshwater habitats by ancestral marine sticklebacks provided the historical events upon which subsequent ecological determinism was contingent. Rather than necessarily being alternative views of how evolution may unfold, sympatric pairs of sticklebacks illustrate how contingency and determinism may act together to promote evolutionary change and help to explain geographical variation in species diversity.

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