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# Evolutionary history of an adaptive radiation in species pairs of threespine sticklebacks (*Gasterosteus*): insights from mitochondrial DNA

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Species pairs of threespine stickleback, *Gasterosteus aculeatus*, co-exist in several lakes in the Strait of Georgia, southwestern British Columbia. One species, 'benthics' is robust-bodied and is morphologically and behaviourally specialized for benthivory. The other species, 'limnetics' is specialized for planktivory in open-water habitats of the lakes. We examined mitochondrial DNA restriction site variation in benthic and limnetic sticklebacks as well as in solitary freshwater, anadromous (sea-run), and marine populations to test: (i) if benthic and limnetic pairs have evolved only once or multiple times (parallel evolution) and (ii) if the species have evolved sympatrically, or allopatrically from 'double invasions' of lakes by ancestral anadromous/marine sticklebacks. Stickleback mtDNA comprised a single clade with a low (mean = 0.40%) degree of sequence divergence among the 77 haplotypes resolved. Most nucleotide diversity (97%) was found within (rather than among) populations of anadromous/marine sticklebacks whereas most diversity (77%) was found among populations in freshwater sticklebacks. Significant differences in haplotype frequencies were found between benthics and limnetics in three of the four species pair lakes examined, but in all cases the pairs within lakes were characterized by unique assemblages of closely related haplotypes. Hierarchical clustering of divergence estimates suggested that comparable species from different lakes have originated independently in all lakes because in no case did comparable species from different lakes cluster together. Divergent species within lakes tended to be more closely related to one another than to species in other lakes and there were two cases were benthics and limnetics within a particular lake were monophyletic. In two of the four two-species lakes, limnetics were less divergent from putative ancestral anadromous/marine stickleback as predicted by the double invasion hypothesis, but in the two other lakes benthics were less divergent. Our data argue strongly that the species pairs have evolved independently in each lake were they now co-exist. Further, in two lakes our data are consistent with the species having evolved by sympatric divergence, but allopatric divergence followed by introgression of mtDNA that has obscured ancestral relationships cannot be discounted completely. Finally, despite remaining uncertainty about the geography of speciation, the species appear to have evolved in the face of gene flow arguing that natural selection acting on trophic ecology has been a major component of ecological speciation in sticklebacks.



ADDITIONAL KEYWORDS:—speciation – zoogeography – genetic variation – gene flow – species pairs.

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## INTRODUCTION

What constitutes a species, and how species originate, are still central issues in evolutionary biology (Dobzhansky, 1937; Mayr, 1963; Otte & Endler, 1989; Rice & Hostert, 1993; Bush, 1994). Two approaches have been commonly employed to study speciation: a vertical or phylogenetic approach and a horizontal or ecological approach. The first involves the reconstruction of the historical relationships among taxa to make inferences on their mode of evolutionary origin (e.g. Meyer *et al.*, 1990; Schliewen, Tautz & Pääbo, 1994; Väinölä, 1995) while the second involves the detailed analysis of the ecological, behavioural, and morphological differences between species in an attempt to understand the processes that drive the speciation process itself (e.g. Coyne & Orr, 1989; McPhail, 1994).

Recently (i.e. late Pleistocene) deglaciated lakes in north temperate freshwater environments harbour a wealth of diversity in the form of undescribed sibling species of fish (reviewed by Taylor, 1999). Sympatric 'species pairs' occur in the families Osmeridae (Taylor & Bentzen, 1993), Salmonidae (Ferguson & Mason, 1981; Pigeon, Chouinard & Bernatchez, 1997) and Gasterosteidae (McPhail, 1984, 1992; Schluter, 1996a,b). Novel ecological opportunities afforded by deglaciation and recolonization from relatively depauperate faunas are thought to be major reasons for the repeated occurrence of such sympatric pairs throughout the northern hemisphere (Hubbs, 1940; Behnke, 1972; Schluter & McPhail, 1992; Schluter, 1996a; Bell & Andrews, 1997). Because these sympatric pairs are of recent origin (i.e. most are thought to be less than 15 000 years old) and perhaps in the final stages of speciation, they represent unique opportunities to combine phylogenetic and ecological approaches to the study of speciation in taxa where reproductive isolation is still evolving (e.g. Bell & Andrews, 1997; Taylor, McPhail & Schluter, 1997).

The threespine stickleback (*Gasterosteus aculeatus*) presents a particularly striking example of a recent adaptive radiation. This gasterosteiform fish is euryhaline, has a Holarctic distribution, and consists of a 'species complex' involving marine, anadromous, and freshwater-resident populations. Although classified under a single latin binomial, the taxonomy of threespine stickleback is remote from evolutionary

reality, and there are many cases of reproductively isolated sticklebacks living in sympatry or parapatry that fulfill the criteria of biological species (see Hagen & McPhail, 1970; McPhail, 1994; Bell, 1995 for discussion of different levels of divergence within *G. aculeatus*).

For example, sympatric, 'species pairs' of sticklebacks are known from six lakes on three different islands in the Strait of Georgia, southwestern British Columbia (Larson, 1976; McPhail, 1984, 1992, 1994). One species in each pair, referred to as 'benthic', is robust-bodied and feeds largely on macrobenthos in littoral habitats and is morphologically and behaviourally specialized for benthivory. The other species in each pair, known as 'limnetic' is terete-shaped, inhabits the limnetic zone of lakes (except during breeding), and is morphologically and behaviourally specialized for planktivory (McPhail, 1984, 1992, 1994; Schluter, 1993). The species show a high degree of microhabitat partitioning during breeding in the littoral habitat, and experimental studies demonstrate clear assortative mating between species under laboratory and field conditions (Ridgway & McPhail, 1984; Nagel & Schluter, 1998). Also, there are consistent biochemical and quantitative genetic differences between sympatric benthics and limnetics (McPhail, 1984, 1994). Another remarkable aspect of adaptive radiation within *Gasterosteus* is that the current distribution of species pairs is apparently limited to recently deglaciated regions in southwestern British Columbia despite the Holarctic distribution of *Gasterosteus aculeatus*. This implies that the radiation has occurred since the Cordilleran ice sheet began to recede about 12 000 years ago and also that there is some unusual feature(s) of southwestern British Columbia that has promoted species pair evolution.

In this study we address two important questions concerning the evolutionary origin of the *Gasterosteus* species pairs. First, does the current geographic distribution of the species pairs represent a single divergence followed by dispersal to the lakes in which they are now sympatric or have the pairs arisen independently multiple times? Second, did the divergence(s) between benthic and limnetic sticklebacks occur via allopatry or did they evolve in sympatry? McPhail (1993) outlined two hypotheses for the origin of benthic and linmetic species pairs. One hypothesis, the 'double invasion' scenario posits that the current species pairs result from two invasions of emerging freshwater habitats by marine/anadromous sticklebacks that colonized lakes following Wisconsinan ice sheet retreat from coastal areas. McPhail (1993) suggested that first invasion resulted in the evolution of a 'benthic-like' solitary stickleback inhabiting lakes because most lakes have extensive, food rich littoral zones and extant solitary lake populations tend to be benthic-like. There is geological evidence that a second marine incursion in coastal areas occurred about 2000 years after the first (Mathews, Fyles & Nasmith, 1970) and McPhail suggested that this second flooding of lowland lakes facilitated a second wave of colonization by marine/anadromous sticklebacks. In lakes already containing benthic-like solitary sticklebacks (as a result of the first colonization event) it is hypothesized that natural selection favoured sticklebacks among the second colonists that could exploit the 'empty' open water niche (McPhail, 1993). Thus, the evolution of this 'limnetic' species was driven by its interaction with the first colonists (i.e. character displacement, Schluter & McPhail, 1992; McPhail, 1993; Taylor, McPhail & Schluter, 1997). A period of allopatry between the two invasions is thought to be central to the evolution of reproductive isolation between stickleback populations during secondary contact following the second invasion. Interestingly, the species pairs are apparently only found in lakes that were subject to two marine floodings; all other lakes contain only single species that exploit littoral habitats, open water habitats, or both

(McPhail, 1993). The alternative model of sympatric speciation posits that the species pairs arose within each lake from a single, common postglacial colonist species perhaps by competitive speciation and disruptive selection (McPhail, 1993; Taylor, McPhail & Schluter, 1997).

The primary objective of our study was to reconstruct the historical relationships of stickleback populations of south-western British Columbia to test the single versus multiple origin hypothesis for the evolution of multiple species pairs in different lakes. A single origin hypothesis predicts that the recovered phylogenetic relationships would show that limnetics from all lakes would form a monophyletic cluster distinct from all benthics that are contained in a second monophyletic grouping. The multiple origin hypothesis predicts that benthics and limnetics should be polyphyletic. Evidence in favour of multiple, independent origins for the species pairs would implicate deterministic factors, natural selection for specialization to alternative trophic niches for example, in the evolution of the species pairs because random factors in evolution would be unlikely to drive independent divergences along parallel ecological axes. Our secondary objective was to test the double invasion versus sympatric speciation scenarios concerning the geography of stickleback speciation. The allopatric double invasion hypothesis predicts that limnetics should be less divergent from ancestral anadromous/marine sticklebacks from which they were more recently derived. Conversely, the sympatric speciation hypothesis predicts that benthics and limnetics within lakes should be monophyletic (decended most recently from a common ancestor after a single invasion by anadromous/marine stickleback) and should be roughly equally divergent from ancestral anadromous/marine sticklebacks (Harrison, 1991; Schluter & McPhail, 1992).

We tested these predictions by conducting a mitochondrial DNA restriction site analysis to reconstruct the historical relationships amongst the various populations under study. Mitochondrial DNA is widely applied in historical reconstructions at the intraspecific level (Avise, 1994; Moore, 1995) and has been used to test sequential colonization hypotheses in a variety of taxa (e.g. Thorpe, McGregor & Jordan, 1994; Ranker, Floyd & Trapp, 1994; Juan, Oromi & Hewitt, 1995; Shaw, 1996).

## MATERIAL AND METHODS

### *Sample collections*

Sticklebacks were collected from 21 lake, river or estuarine systems (Table 1; Fig. 1) mostly from southwestern British Columbia, but ranging to southern California. These samples included four cases of sympatric benthic-limnetic species pairs (Enos, Paxton, Priest, and Emily lakes), three pairs of parapatric anadromous and stream-resident populations, eight allopatric anadromous populations, and nine solitary freshwater populations. Fish were collected with minnow traps or pole seines. Liver, spleen, heart, and muscle tissues were taken and placed in 95% ethanol where they were stored at 4°C until DNA extraction.

### *mtDNA assays*

Genomic DNA was was obtained using Pronase digestion and phenol/chloroform extraction methods as outlined in Taylor, Foote & Wood (1996). Typically, 2–5 $\mu$g

TABLE 1. Haplotype diversity (with SE) and nucleotide diversity ($\times 100$) of threespine stickleback collected in the study

| Population | Sample size | Sample year | Haplotype diversity | Nucleotide diversity |
|---|---|---|---|---|
| SOLITARY LAKES | | | | |
| Tremerton Lake | 15 | 1994 | 0.0000 (0.0000) | 0.0000 |
| Ogden Lake | 15 | 1994 | 0.0000 (0.0000) | 0.0000 |
| Brannon Lake | 17 | 1995 | 0.4359 (0.0963) | 0.2677 |
| Nanaimo Lake | 15 | 1994 | 0.0000 (0.0000) | 0.0000 |
| Kennedy Lake | 13 | 1994 | 0.7843 (0.0836) | 0.3094 |
| Cranby Lake | 23 | 1994 | 0.7382 (0.0258) | 0.3859 |
| Otter Point Slough | 10 | 1995 | 0.0000 (0.0000) | 0.0000 |
| SYMPATRIC LAKE PAIRS | | | | |
| Paxton Lake (limnetics) | 17 | 1994 | 0.3290 (0.1207) | 0.0885 |
| Paxton Lake (benthics) | 16 | 1994 | 0.0000 (0.0000) | 0.0000 |
| Priest Lake (limnetics) | 15 | 1994 | 0.7196 (0.0763) | 0.3443 |
| Priest Lake (benthics) | 15 | 1994 | 0.8985 (0.0303) | 0.3653 |
| Emily Lake (limnetics) | 18 | 1995 | 0.0000 (0.0000) | 0.0000 |
| Emily Lake (benthics) | 18 | 1995 | 0.6989 (0.0835) | 0.3240 |
| Enos Lake (limnetics) | 14 | 1994 | 0.6943 (0.0844) | 0.2296 |
| Enos Lake (benthics) | 14 | 1994 | 0.8042 (0.0453) | 0.2216 |
| Enos Lake (benthics) | 28 | 1995 | 0.5844 (0.0622) | 0.1981 |
| STREAM-RESIDENTS | | | | |
| Salmon River | 28 | 1994 | 0.0000 (0.0000) | 0.0000 |
| Little Campbell River | 14 | 1994 | 0.7090 (0.0474) | 0.3074 |
| Big Beef Creek | 19 | 1996 | 0.8116 (0.0456) | 0.5192 |
| Ventura River | 15 | 1996 | 0.0000 (0.0000) | 0.0000 |
| Sespe River | 15 | 1996 | 0.1376 (0.0837) | 0.0136 |
| ANADROMOUS/MARINE POPULATIONS | | | | |
| Little Campbell River | 16 | 1994 | 0.8092 (0.0577) | 0.4229 |
| Salmon River | 29 | 1994 | 0.3146 (0.0788) | 0.1944 |
| Cranby Creek | 16 | 1994 | 0.8627 (0.0353) | 0.4108 |
| Nanaimo River | 15 | 1994 | 0.7080 (0.0865) | 0.4099 |
| Witty's Lagoon | 19 | 1995 | 0.8421 (0.0487) | 0.4196 |
| French Creek | 16 | 1994 | 0.6087 (0.0867) | 0.3925 |
| Oyster Lagoon | 14 | 1994 | 0.8629 (0.0418) | 0.4301 |
| Salt Lagoon | 14 | 1994 | 0.7937 (0.0666) | 0.4390 |

of genomic DNA was digested overnight with 12 endonucleases: three multipentameric (*Bst*N I, *Nci* I, and *Sty* I), five multihexameric (*Ava* I, *Ava* II, *Ban* I, *Hae* II, and *Hin*c II) and four hexameric (*Dra* I, *Hin*d III, *Pst* I, and *Pvu* II) enzymes. Digestion conditions were as recommended by the manufacturer (New England Biolabs). Restricted DNAs were electrophoresed on 0.8–1.2% agarose gels, and Southern blotted under vacuum as detailed by Taylor *et al.* (1996).

Mitochondrial DNA restriction fragment variation was assayed by hybridization of membrane-bound stickleback DNA with digoxigenin-labelled threespine stickleback mtDNA cloned into PUC 19. Hybridization conditions (58°C, final wash stringency, $2 \times$ SSC/0.1% SDS at 58°C) and detection of probe-stickleback DNA hybrids by chemiluminesence are detailed in Taylor *et al.* (1996).

### *Data analyses*

All restriction fragment length polymorphisms (RFLPs) in *Gasterosteus* could be accounted for by single or double restriction site changes. Consequently, a presence/

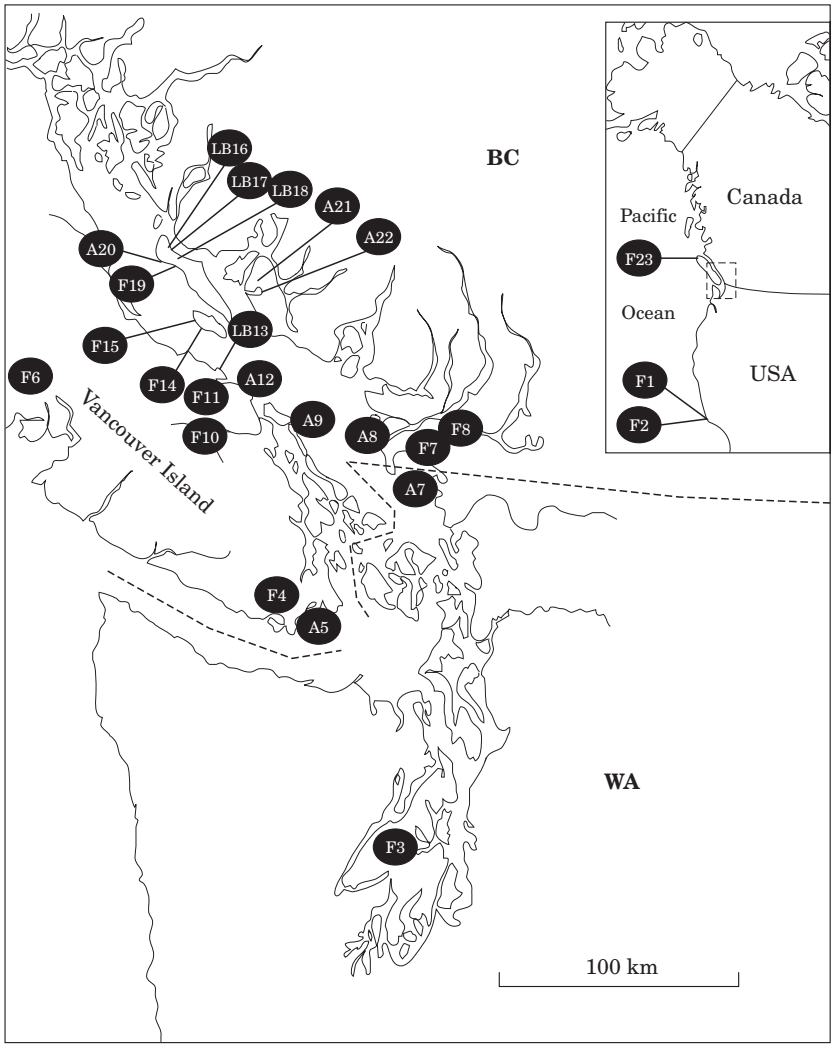


Figure 1. Distribution of populations of threespine stickleback sampled for mtDNA analysis. Inset shows position of enlarged area in western North America. A = Anadromous/marine, F = lake or stream, L = limnetic, and B = benthic sticklebacks, respectively. 1 = Ventura River, 2 = Sespe River, 3 = Big Beef Creek, 4 = Otter Point Slough, 5 = Witty's Lagoon, 6 = Kennedy Lake, 7 = Little Campbell River, 8 = Salmon River, 9 = Nanaimo River, 10 = Nanaimo Lake, 11 = Brannon Lake, 12 = French Creek, 13 = Enos Lake, 14 = Ogden Lake, 15 = Tremerton Lake, 16 = Emily Lake, 17 = Priest Lake, 18 = Paxton Lake, 19 = Cranby Lake, 20 = Cranby Creek, 21 = Salt Lagoon, 22 = Oyster Lagoon, 23 = Misty Lake.

absence restriction site matrix was contructed for each RFLP observed for each enzyme and was given a single capital letter code (e.g. *Ava* I A, B, C, etc.). Each fish was then characterized by a 12 letter composite haplotype code (Table 2); each letter representing the restriction site code for each of the 12 enzymes (e.g. AAAAAAAAAAAA, ABAAAAAAAAAA, etc). A site matrix for each of the composite haplotypes resolved was constructed using the program REAP (McElroy *et al.*, 1992). The composite haplotype restriction site matrix so generated formed the basis for

TABLE 2. Composite haplotypes of threespine stickleback mtDNA resolved with 12 enzymes and their percentage frequency. Haplotype 78 was not found in the study populations, but was used for comparative purposes (see text for details). Each letter represents the restriction fragment length polymorphism resolved with *Ava* I, *Ava* II, *Ban* II, *Bst*U I, *Dra* I, *Hae* II, *Hin*c II, *Hin*d III, *Nci* I, *Pst* I, *Pvu* II, and *Sty* I, respectively

| Haplotype number | Haplotype code | Percentage frequency | Haplotype number | Haplotype code | Percentage frequency |
|---|---|---|---|---|---|
| 1 | AAACAADAAAAA | 6.36 | 41 | AADCAABAAAAA | 0.21 |
| 2 | ABAAAADAAAAA | 0.64 | 42 | AGADAAAAAAAD | 0.42 |
| 3 | AAACAAFAAAAB | 0.64 | 43 | ACAAAAEABAAA | 0.42 |
| 4 | ACADAAEAAAAA | 0.21 | 44 | AAAEAAAAAAAA | 0.21 |
| 5 | ACACAAEABAAA | 0.42 | 45 | ACACAAAAAAAA | 0.21 |
| 6 | AACCAAAAAAAB | 0.21 | 46 | AAAFAACAAAAA | 0.21 |
| 7 | ACAAAAEAAAAA | 8.68 | 47 | AAACAACAAAAC | 0.21 |
| 8 | ACBAAAEAAAAA | 0.84 | 48 | AAACAACAAAAA | 0.42 |
| 9 | AAACAABAAAAB | 0.64 | 49 | AAAJAAAAAAAA | 0.42 |
| 10 | ACAABAECAAAA | 0.42 | 50 | AAAKAAAAAAAA | 0.21 |
| 11 | ACABAAEAAAAA | 0.63 | 51 | AIACAAAAAAAA | 0.21 |
| 12 | AAACAAAAAAAA | 25.8 | 52 | ABACAAAAAAAA | 0.42 |
| 13 | AFACAAEAAAAA | 0.64 | 53 | AAACAAAAEAAA | 0.21 |
| 14 | ADACAAEAAAAA | 2.75 | 54 | AIAKAAAAAAAA | 4.67 |
| 15 | AEACAAEAAAAA | 3.81 | 55 | ACAAAAGAAAAA | 1.06 |
| 16 | ACAEAAEAAAAA | 1.06 | 56 | ACAAAAHAAAAA | 1.69 |
| 17 | ACCCAAEAAAAC | 0.64 | 57 | ACAAAAHAAADA | 0.63 |
| 18 | ACACAAEAAAAA | 0.85 | 58 | AAADAABBAAAA | 0.21 |
| 19 | ACACAAEACAAA | 0.21 | 59 | AAACBAAAAAAA | 0.42 |
| 20 | AAAGAAAAAAAA | 0.21 | 60 | AGACAACAAAAA | 0.21 |
| 21 | AAAFAABAAAAA | 0.42 | 61 | AAALAAAAGAAA | 0.21 |
| 22 | AAACCABAAAAA | 0.21 | 62 | AAADAABAAAAA | 0.42 |
| 23 | AAACAABAAAAA | 4.67 | 63 | AJACAAAAAAAA | 0.42 |
| 24 | ABACAABAAAAA | 0.21 | 64 | AAACAABAHAAA | 0.21 |
| 25 | AHACAABAAAAA | 0.21 | 65 | AAAMAABAFAAA | 0.21 |
| 26 | AGACAAAAAAAA | 0.42 | 66 | AAABAAAAAAAA | 2.75 |
| 27 | AAADAAAAAAAA | 0.64 | 67 | BIECAAAAAAAA | 0.21 |
| 28 | AAADAAAAAAAC | 0.21 | 68 | AAACAAAABAAA | 0.21 |
| 29 | ACAEAAEAAABA | 0.21 | 69 | AAACAAAAAAAB | 1.06 |
| 30 | AAAFAAAAAAAA | 0.21 | 70 | AEAAAAEAAAAA | 0.42 |
| 31 | AIABAAAAAAAA | 0.21 | 71 | ACFAAAEAAAAA | 0.21 |
| 32 | AAAHAAAAAAAA | 0.85 | 72 | AMACAAEAAAAA | 6.14 |
| 33 | AJACAABADAAA | 0.21 | 73 | AMACAAJAAAAA | 0.21 |
| 34 | AAACAAEAAAAA | 0.85 | 74 | AEACAAAAKAAA | 0.64 |
| 35 | AKACAAAAAAAA | 0.85 | 75 | AAAOAAAAAAAA | 3.18 |
| 36 | AAAIAAAAAAAA | 1.27 | 76 | ACACAAFAAAAC | 0.21 |
| 37 | AGCCAAAAAAAA | 0.42 | 77 | ACAAAAEAIAAA | 0.21 |
| 38 | AACCAAAAAAAA | 1.06 | 78 | BLCNDBIAJBAA | 0.00 |
| 39 | AAACAAFAAAAA | 1.27 | | | |
| 40 | AADCAABAAACA | 0.21 | | | |

subsequent analyses of haplotype and nucleotide diversity using programs found in REAP.

Divergence among haplotypes was estimated as the number of nucleotide substitutions per site, *d* (Nei & Miller, 1990). The restriction site matrix was also organized into a phylogenetic tree using Wagner parsimony. Confidence in the branches separating groups of haplotypes was assessed by bootstrap resampling of the restriction site matrix with 1000 replications and using the programs SEQBOOT, MIX, and CONSENSE of PHYLIP (version 3.5, Felsenstein 1993). Bootstrap analysis of the haplotype divergence matrix was completed by using BOOT ( Jaarola

& Tegelström, 1995). Replicate divergence estimates were clustered using the Neighbour-Joining algorithm (Saitou & Nei, 1987).

Stickleback mtDNA in the North Pacific has been shown to consist of two major clades that differ in sequence by about 2.5% (O'Reilly *et al.*, 1993; Ortí *et al.*, 1994; Thompson, Taylor & McPhail, 1997). The two clades ('Japanese' (Ortí *et al.*, 1994) or 'Trans-North Pacific Clade' (TNPC, Thompson *et al.*, 1997) and the 'Euro-North American Clade' (ENAC, Ortí *et al.*, 1994)) broadly overlap in distribution from Japan to northern Vancouver Island (O'Reilly *et al.* 1992; Ortí *et al.*, 1994; Thompson *et al.*, 1997), but only the ENAC has been found in sticklebacks sampled from south of northeastern Vancouver Island (Ortí *et al.*, 1994; Taylor, unpublished data). We included the composite haplotype resolved with the 12 enzymes listed above from fish representative of the mtDNA TNPC to serve as an outgroup in our phylogenetic analyses. Sticklebacks of the TNPC were collected from an inlet stream of Misty Lake on northeastern Vancouver Island (Fig. 1) and were previously shown by Thompson *et al.* (1997) to carry TNPC mtDNA.

Total nucleotide diversity was partitioned into variance components, and their corresponding phi statistics, by using the Analysis of Molecular Variance (AMOVA) approach of Excoffier, Smouse & Quattro (1992). In this analysis, our highest hierarchical grouping was a distinction between 'freshwater' and 'anadromous/marine' populations ($\varphi_{CT}$), followed by variation among populations within each of these life-history groups ($\varphi_{SC}$), and finally, variation within individual populations ($\varphi_{ST}$). The statistical significance of each of the variance components was estimated by random permutations of the original restriction site and haplotype frequency matrices (Excoffier *et al.*, 1992).

The statistical significance of differences in haplotype frequency distribution between sympatric species pairs was assessed by chi-square randomization procedure of Roff & Bentzen (1989) as implemented in REAP. Haplotype frequency distributions within populations were used in conjunction with estimates of $d$ to estimate nucleotide diversity, $\pi$, within populations (Nei & Tajima, 1981). Nucleotide divergence between pairs of populations was defined as the residual divergence not explained by diversity within populations (Nei & Miller, 1990). Estimates of nucleotide divergence among populations were used to estimate the relationships among populations using the Neighbour-Joining algorithm. Relationships among populations were also assessed using the 'hierarchical analysis' of Holsinger & Mason-Gamer (1996). This analysis calculates $\hat{g}_{ST}$ which is the proportion of nucleotide diversity in the sample that can be attributed to differences among populations (Holsinger & Mason-Gamer, 1996). This statistic is calculated for each pair of populations or between paired groups of populations and is accompanied by a clustering algorithm that can test the statistical significance of branch points in the resulting dendrogram (Holsinger & Mason-Gamer, 1996). The hierarchical analysis was completed using the program NUCLEODIV (Holsinger & Mason-Gamer, 1996).

Our phylogenetic analyses assume that the mitochondrial DNA variation is selectively neutral and, therefore, marks ancestral relationships. To assess this assumption, we tested for evidence of natural selection organizing the restriction site variation using the statistical procedure of Tajima (1989a). This procedure involves calculation of the $D$ statistic, a measure of the difference between the number of polymorphic sites, $S$, and the average number of (pairwise) site differences between haplotypes, $\hat{k}$. Both $S$ and $\hat{k}$ are estimates of $M$, essentially the level of neutral genetic variation expected in a population at drift/mutation equilibrium

(Tajima, 1989a). Any difference between $S$ and $\hat{k}$ may, therefore, signal the effects of selection on certain deleterious mutant haplotypes which influence the estimates of $S$, but not $\hat{k}$ (Tajima, 1989a). The neutral mutation hypothesis, therefore, predicts that $D$ should be 0. The Tajima test assumes that the populations being tested have had large and stable sizes, an assumption that is likely violated in our case particularly for freshwater sticklebacks. Violation of this assumption, however, tends to exaggerate $D$ values (Tajima, 1989b) which would result in *higher* probabilities of *falsely* rejecting the null hypothesis of neutrality.

## RESULTS

### *Restriction site variation and selection*

A total of 129 restriction sites representing 525 base pairs were assayed in our survey of mtDNA from 475 *Gasterosteus*. Employing Tajima's (1989a) statistical procedure within each population, the $D$ values obtained ranged from $-2.12$ to 1.25. All values calculated were not significantly different from 0 when adjusted for multiple tests (i.e. tablewide $P>0.05$, Rice (1989)). These tests, therefore, indicated that the neutral mutation hypothesis cannot be rejected as an explanation for the observed restriction site variation.

### *Haplotype diversity and sequence analysis*

Our sampling of the mtDNA genome resolved 77 composite haplotypes distinguished by between 1 and 8 restriction site changes (mean = 4.1, data matrix available from EBT). Overall, nucleotide divergence among haplotypes was fairly low with an average ($\pm$SE) of $0.39 \pm 0.02\%$ (range 0.01–0.8%). When fish from the divergent Trans-North Pacific Clade (TNPC) were also examined, a further haplotype was resolved that differed by 20–26 restriction sites from the remaining 77 haplotypes from southern B.C., Washington, and California (sequence divergence = 2.0–2.8%). The latter stickleback haplotypes share restriction sites diagnostic for a southern clade of sticklebacks (originated named the 'Marine Lineage' by O'Reilly *et al.* (1993)) and which distinguish them from TNPC sticklebacks (e.g. unique site gains at *Hin*c II and *Pvu* II). This southern lineage was subsequently redefined by more extensive geographic sampling and cytochrome *b* sequencing and RFLP analyses as the 'Euro-North American Clade' (ENAC) by Ortí *et al.* (1994). We confirmed the identity of representative fish in our study (exclusive of the TNPC haplotype) as members of this southern lineage or ENAC by the PCR-RFLP test of Ortí *et al.* (1994); the two most divergent haplotypes in our study all possessed the *Nsi* I restriction site diagnostic for the ENAC (Ortí *et al.*, 1994; Deagle, Reimchen & Levin, 1996).

Both Wagner parsimony and Neighbour-Joining analyses of nucleotide divergence among haplotypes using the TNPC haplotype as the outgroup (Fig. 2) suggested the existence of two groups of haplotypes within the southern, or ENAC, assemblage of haplotypes. These putative groupings were centered roughly on haplotypes 10 and 21 and differed from each other by about 0.48% in sequence. Bootstrap resampling of the restriction site matrix in parsimony and distance-based analyses,

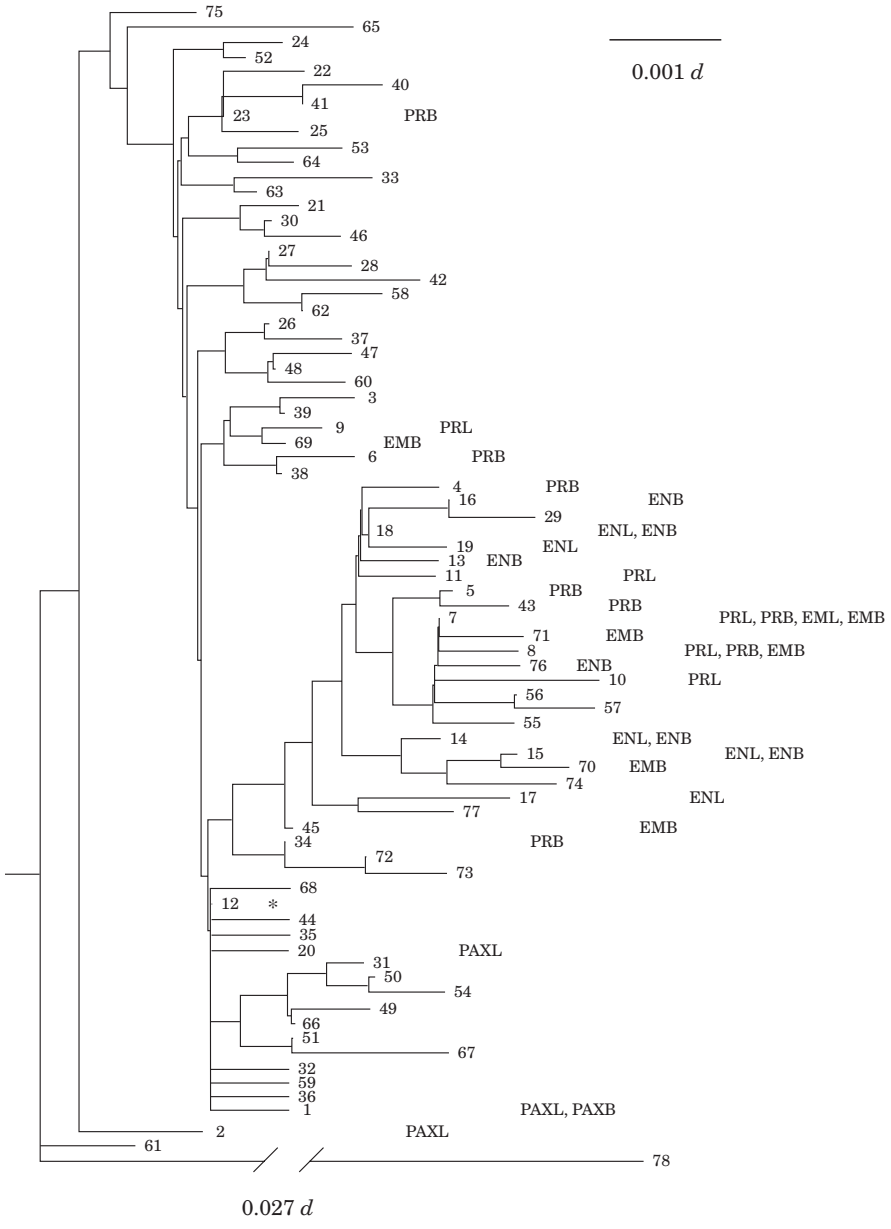


Figure 2. Neighbour-Joining tree (rooted at haplotype 78) of relationships among mtDNA composite haplotypes of threespine sticklebacks sampled from southwestern British Columbia, Washington, and California. Tree was derived from estimates of mtDNA nucleotide divergence among haplotypes. Also indicated is the distribution of haplotypes among sticklebacks from two species lakes; PRL = Priest Lake limnetic, PRB = Priest Lake benthic, ENL = Enos Lake limnetic, ENB = Enos Lake benthic, PL = Paxton Lake limnetic, PB = Paxton Lake benthic, EML = Emily Lake limnetic, EMB = Emily Lake benthic. Haplotype 12 (*) was found in approximately 25% of all stickleback and represents the most widespread haplotype.

however, suggested this apparent bifurcation of haplotype groups was tenuous as the node separating putative major groups in Figure 2 was not recovered in the consensus trees. In fact, only near terminal pairs of haplotypes ($n=6$) were resolved in more than 50% of the bootstrap replicates. Further, there were no fixed restriction sites that discriminated major groups of mtDNA and the full data set of pairwise sequence divergences approximated a normal distribution with a single mode at about 0.4%.

### *mtDNA diversity in freshwater and marine populations*

Analysis of the nucleotide diversity using AMOVA indicated that 13% of the variation was attributable to differences between freshwater and anadromous/marine life-history groupings of sticklebacks ($\varphi_{CT}=0.130$, $P=0.007$), 55% was attributable to variation among populations within these life-history groups ($\varphi_{SC}=0.629$, $P<0.001$), and 32% was attributable to variation within populations ($\varphi_{ST}=0.667$, $P<0.001$). When the data were analysed separately by life-history group, distinct patterns of genetic variation were resolved; 97% of the nucleotide variation was found within populations of anadromous/marine populations with only 3% occurring among populations ($\varphi_{ST}=0.034$, $P<0.001$), whereas in freshwater populations 23% of the variation occurred within populations and 77% occurred among populations ($\varphi_{ST}=0.769$, $P<0.001$). Greater within-population variation in anadromous/marine populations was also reflected in measures of haplotype and nucleotiode diversity (Table 1). Marine/anadromous populations' haplotype and nucleotide ($\times 100$) diversities averaged (SE) 0.725 (0.066) and 0.390 (0.028), respectively whereas these measures in freshwater populations averaged 0.385 (0.082) and 0.174 (0.039), respectively and several freshwater populations were fixed for alternative haplotypes (Table 3).

### *Divergence between sympatric populations*

Levels of haplotype frequency differentiation and nucleotide divergence between sympatric species pairs of sticklebacks differed markedly in the four two-species lakes (e.g. Table 3, Fig. 3). In Enos Lake, benthic sticklebacks collected in 1994 and 1995 had haplotype frequencies that were statistically indistinguishable ($P=0.69$, Table 3) suggesting that haplotype frequencies are temporally stable. Sequence divergence between sympatric species was estimated as 0.000%, 0.006%, 0.016%, and 0.06% for Paxton, Enos,Priest, and Emily lakes, respectively. Within each of Emily, Priest, and Enos lakes, benthic and limnetic sticklebacks had significantly different haplotype frequencies (maximum $P=0.02$), but the species in Paxton Lake did not ($P=0.1$). The four lakes with sympatric stickleback species were characterized by each lake comprising an almost unique assemblage of mtDNA composite haplotypes (Table 3; Fig. 2). Only two of the 27 haplotypes found in two-species lakes were found in more than one of these lakes; haplotypes 7 and 8 were found both in Emily and Priest lakes. The non-overlapping haplotype distributions found between comparable species in different lakes was also reflected in the results of an AMOVA analysis that grouped lakes with two species by phenotype. There was no significant variation attributable to phenotype (i.e. all limnetics grouped separately from all benthics,

TABLE 3. Distribution of mtDNA composite haplotypes among threespine stickleback populations. Numbers represent composite haplotypes defined in Table 2, parenthetical values represent their absolute frequency. Haplotypes not accompanied by parenthetical values represent single observations

| Population | Haplotypes present |
|---|---|
| SOLITARY LAKES | |
| Tremerton Lake | 75 (15) |
| Ogden Lake | 15 (15) |
| Brannon Lake | 1, 12 (15), 34 |
| Nanaimo Lake | 54 (15) |
| Kennedy Lake | 12, 23 (2), 38 (2), 54 (6), 67, 68 |
| Cranby Lake | 7 (7), 55 (5), 56 (8), 57 (3) |
| Otter Point Slough | 66 (10) |
| SYMPATRIC LAKE PAIRS | |
| Paxton Lake (limnetics) | 1 (13), 2 (3), 20 |
| Paxton Lake (benthics) | 1 (16) |
| Priest Lake (limnetics) | 7 (7), 8 (2), 9, 10 (2), 11, (3) |
| Priest Lake (benthics) | 3 (3), 4, 5 (2), 6, 7, 8, 9 (2), 23, 34, 43 (2) |
| Emily Lake (limnetics) | 7 (18) |
| Emily Lake (benthics) | 7 (8), 8, 69 (5), 70 (2), 71, 77 |
| Enos Lake (limnetics) | 14 (8), 15, 17 (3), 18, 19 |
| Enos Lake (benthics, 1994) | 13 (2), 14 (5), 15 (2), 16, 18 (2), 76 (1) |
| Enos Lake (benthics, 1995) | 13 (3), 14 (17), 15 (2), 16, 18 (2), 76 (6) |
| STREAM-RESIDENTS | |
| Salmon River | 12 (28) |
| Little Campbell River | 12, 35 (4), 36 (6), 39 (3) |
| Big Beef Creek | 12 (9), 23 (3), 52, 62, 66 (2), 74 (3) |
| Ventura River | 72 (15) |
| Sespe River | 72 (14), 73 |
| ANADROMOUS/MARINE POPULATIONS | |
| Little Campbell River | 12 (6), 23, 37 (2), 38, 39 (2), 40, 41, 42 (2) |
| Salmon River | 12 (24), 23, 27, 31, 32, 33 |
| Cranby Creek | 12 (5), 18, 21 (2), 22, 23 (3), 24, 25, 26 (2), 30 |
| Nanaimo River | 12 (8), 23, 32, 34, 50, 51, 52, 53 |
| Witty's Lagoon | 12 (7), 23 (2), 27, (2), 28, 29, 34, 39, 44, 45, 46, 47 |
| French Creek | 12 (7), 23 (5), 48 (2), 49 (2) |
| Oyster Lagoon | 12 (5), 23, 32, 38, 54, 58, 59, 60, 61, 62 |
| Salt Lagoon | 12 (6), 23 (2), 32, 59, 63, 64, 65, 66 |

($\varphi_{CT} = -0.140$, $P = 0.74$), but variation attributable to differences between sympatric species within lakes ($\varphi_{SC} = 0.690$, $P < 0.001$) and within species among lakes ($\varphi_{ST} = 0.45$, $P < 0.001$), accounted for all the variability. Finally, in two of the four two-species lakes, benthics were characterized by higher levels of haplotype and nucleotide diversity (Table 1).

### *mtDNA phylogeny of stickleback populations*

The nucleotide divergence estimates among populations averaged 0.15% (range 0.0–0.68%) and were organized into a population phylogeny by the algorithm of Holsinger & Mason-Gamer (1996; Fig. 3). This analysis indicated that comparable species in different lakes were not monophyletic (i.e. they did not cluster together). The only exception involved Priest and Emily lakes limnetics which formed an unresolved grouping with Emily Lake benthics and a nearby solitary freshwater population (Cranby Lake). Further, both in Paxton and Enos lakes, benthic and

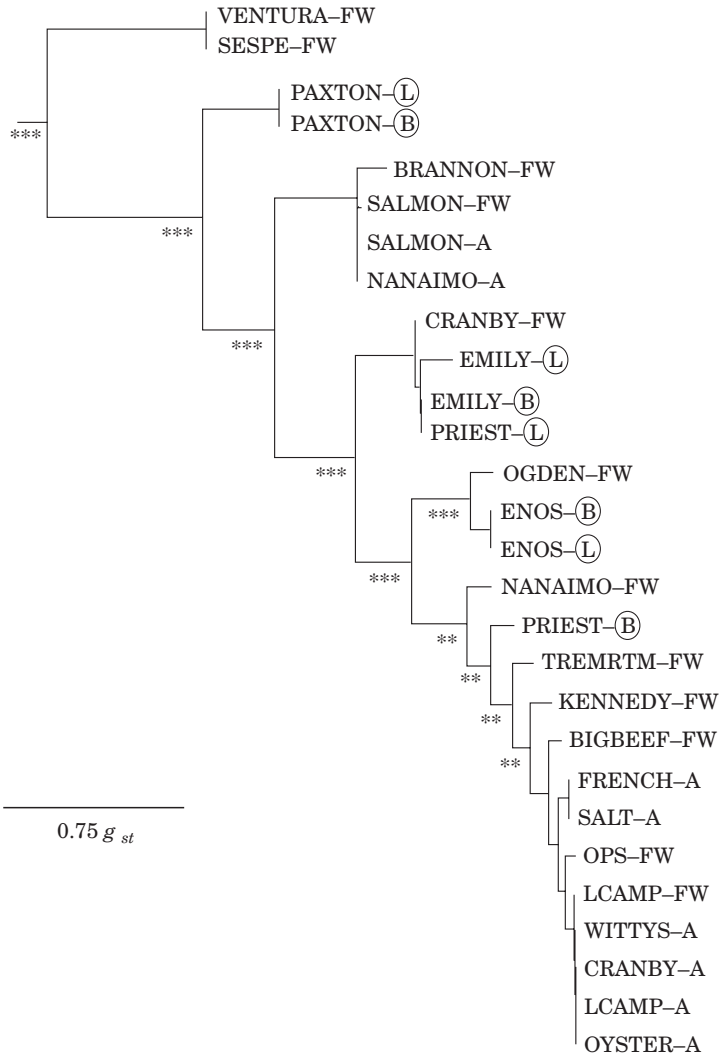


Figure 3. Phenogram of $g_{ST}$ among populations following the analysis of Hoslinger and Mason-Gamer (1996). Asterisks at branch points represent statistical significance (*** = $P$<0.001, ** = $P$<0.01) of branch lengths separating decendent groups. A = Anadromous/marine, FW = lake or stream, L = limnetic, and B = benthic sticklebacks, respectively.

limnetic species within lakes formed monophyletic groups that were statistically distinguishable in the dendrogram from each other and the species pairs in Priest and Emily lakes (Fig. 3). Most solitary freshwater populations were found in clusters distinct from anadromous/marine populations most of which formed an unresolved grouping of six populations (Fig. 3). The permutation analyses of branch lengths coupled with the hierarchical clustering analysis suggested the existence of several distinct groupings of populations (Fig. 3). For instance, the two California populations, the species pair in Paxton Lake, the pair in Emily Lake and Priest Lake limnetics, the Enos Lake pair (plus one solitary population), and Priest Lake benthics (plus several solitary and anadromous/marine populations) were groupings all separable

from one another by statistically significant branch lengths (all $P<0.0001$, Fig. 3). These patterns strongly suggest that the pairs have arisen independently in each of the lakes where they occur because benthic and limnetic sticklebacks from three of the four two-species lakes were found in 'lake-specific' clusters that were statistically distinguishable from one another (i.e. compare positions of Paxton, Emily, and Enos lakes' species pairs, Fig. 3). A similar set of relationships was also resolved when net sequence divergence estimates were clustered by Neighbour-Joining (dendrogram not shown); comparable species in different lakes did not cluster together, rather divergent pairs within lakes tended to form groupings distinct from species pairs in other lakes.

As predicted by the double invasion hypothesis, limnetic sticklebacks from Paxton and Enos lakes were marginally less divergent from the various marine/anadromous populations than were benthic sticklebacks from those lakes ($d \times 100 = 0.18$ (SE = 0.06) and 0.08 (0.07) versus 0.23 (SE = 0.07) and 0.09 (0.06), respectively). The reverse situation, however, held for comparisons between limnetics or benthics and anadromous/marine fish for Emily and Priest lakes ($d \times 100 = 0.33$ (SE = 0.09) and 0.12 (SE = 0.07) for limnetics versus 0.09 (SE = 0.06) and 0.07 (SE = 0.06) for benthics.

## DISCUSSION

### *Parallel evolution of stickleback species pairs*

The mechanisms that produce new species are still controversial in evolutionary biology and two important questions remain unresolved: (i) the geography of speciation, i.e. does non-allopatric speciation such as sympatric or parapatric speciation occur in nature? and (ii) what forces drive divergence between a new species and its ancestor (genetic drift, founder events, or natural selection)? Within the *Gasterosteus aculeatus* species complex, adaptive variation exists at a variety of levels (Bell, 1994, 1995; McPhail, 1994); however, within this complex the divergence of the benthic and limnetic sympatric species pairs is thought to represent a case of rapid speciation. Previous work on the pre-reproductive and reproductive ecology of these pairs indicate that sympatric limnetic and benthic sticklebacks behave as biological species (*sensu* Mayr, 1963; McPhail, 1984, 1992). For two reasons, our mtDNA evidence, in concert with these earlier studies, implicate deterministic factors, such as natural or sexual selection, as important factors driving the divergence of the pairs. First, the benthic and limnetic species in each of the four lakes appear to have arisen independently because the pairs in each lake are characterized by a unique assemblage of mtDNA haplotypes (Fig. 2; Table 3). Second, neither the benthics nor limnetics form monophyletic groups among lakes. Instead, in two lakes the divergent forms are monophyletic (Fig. 3) and in the other lakes divergent forms tend to cluster more closely with one another than either species does with comparable species in other lakes (Fig. 3). In addition, our AMOVA results indicated that there was no significant partitioning of the mtDNA variation by phenotype; 'limnetics' and 'benthics' in different lakes do not constitute natural groups. These results would not be expected if benthics and limnetics had diverged only once and subsequently colonized the different lakes in which they are now sympatric. Thus, our data

strongly suggest that the species pairs arose independently in each lake presumably through parallel evolution (cf. Simpson, 1944; Bell *et al.*, 1993; Schluter & McPhail, 1993; Taylor, Foote & Wood, 1996; Pigeon, Chouinard & Bernatchez, 1997).

Ecologically, the pairs in each lake have diverged along identical axes; the limnetic species forages as a planktivore in open water while the benthic species exploits the littoral zone and feeds largely on macrobenthos (Larson, 1976; Bentzen & McPhail, 1984; Schluter, 1993). As well benthics and limnetics in each lake differ morphologically in traits that have been clearly demonstrated to be functionally related to differences in feeding performance in different habitats (Bentzen & McPhail, 1984; Schluter, 1993, 1995). Further, the benthic and limnetic species in different lakes exhibit strikingly similar differences in reproductive behaviour and microhabitat selection (Ridgway & McPhail, 1984, Hatfield & Schluter, 1996). These replicate divergences between the pairs in different lakes are unlikely to have arisen randomly and, in combination with our mtDNA evidence for the independent origins of each pair, strongly argue that divergent selection for specialization in alternative ecological niches is a major factor promoting divergence between benthic and limnetic sticklebacks. Such inferences are supported by Schluter (1994) who provided experimental evidence for the importance of feeding ecology and competition for trophic resources as mechanisms driving morphological differentiation in stickleback populations.

Recently, Thompson *et al.* (1997) demonstrated that the ecological divergence of parapatric 'lake' and 'stream' forms of sticklebacks also arose independently in mtDNA lineages that showed 2–3% sequence divergence, levels of divergence that probably predate the Pleistocene. Our results indicate that similar ecological divergences can occur within single lineages (mean pairwise sequence divergence of about 0.4%). Further, we have documented low levels of sequence divergence between the benthic-limnetic species pairs within each lake (*d* from 0.000 to 0.06%). Such small divergences stand in marked contrast to much higher levels of divergence reported for some sympatric populations of lake whitefish (Bernatchez & Dodson, 1990) and brown trout (Ferguson & Mason, 1981; Hynes, Ferguson & McCann, 1996). In whitefish and brown trout, these large differences have been interpreted as evidence of secondary contact between lineages that have diverged after long periods (i.e. pre-Wisconsinan) of allopatric isolation. Our data, therefore, argue for much more recent, postglacial (i.e. within the last 12 000 years) evolution of species pairs in sticklebacks. Recent divergences, however, do not exclude microallopatric isolation as a factor in species pair evolution. Microallopatric isolation may have occurred frequently during deglaciation as ice sheets shifted, sea levels fluctuated, and/or local watershed flow patterns changed (Behnke, 1972). In aggregate, the multifaceted data collected for benthic and limnetic pairs of threespine sticklebacks provide evidence that divergence to the level of biological species may be driven by natural selection and can proceed rapidly. Schluter (1996a) reviewed similar evidence for a variety of north temperate freshwater fishes, chiefly in the families Gasterosteidiae, Salmonidae, and Osmeridae (see also Bell & Andrews, 1997; Taylor, 1999). He observed a striking concordance in the ecological axis of divergence among these groups that strongly implicates trophic ecology as a primary factor in promoting divergence in sympatry (but see Chouinard, Pigeon & Bernatchez, 1996; Taylor *et al.*, 1997). This concordance in a variety of taxa across a broad geographic range argues that ecological factors are a major mechanism of evolutionary divergence.

*Geography of speciation in sticklebacks*

McPhail (1993) outlined two hypotheses for the origin of the benthic and limnetic species pairs: an allopatric 'double invasion' hypothesis, and a sympatric speciation hypothesis. Both hypotheses assume that freshwater sticklebacks were derived from anadromous/marine fish that colonized freshwater habitats during deglaciation about 12 000 years ago. Our mtDNA data support this assumption. For instance, anadromous/marine populations have haplotype and nucleotide diversities that are about twice as great as those of freshwater lake and stream populations, and the most common haplotype (No. 12) was found in all anadromous/marine populations and several freshwater populations (Table 3). In addition, our AMOVA analysis indicated that much greater variation was found among freshwater populations (77% of total) than among anadromous populations (3% of total). These observations are consistent with derivation of freshwater stickleback from anadromous/marine colonists that over time have maintained larger evolutionary effective population sizes (Avise, Ball & Arnold, 1988). By contrast, the molecular diversity in freshwater populations has likely been reduced by founder effects during colonization and subsequent bottlenecks (including, perhaps, those induced by selection for survival in freshwater habitats) in relatively small lake and stream populations (cf. Ward, Woodwark & Skibinski, 1994; Ovenden & White 1990; Brawn *et al.*, 1996; Strecker *et al.*, 1996). The reduced genetic variation within founding populations relative to larger source populations is consistent with theoretical expectations (Nei, Maruyama & Chakraborty, 1975; Hewitt, 1996), was predicted for sticklebacks invading freshwater by Bell (1976), and has been observed in earlier allozyme studies assays of sticklebacks (Withler & McPhail, 1985) and more recently with microsatellites (E.B. Taylor, unpubl. data).

The multiple, independent origins of sympatric pairs of sticklebacks may have resulted from repeated divergences within a double invasion scenario for each lake. Multiple invasion of freshwater habitats by euryhaline fishes has been suggested as a common phenomenon and mechanism of divergence in temperate areas (Svardson, 1961; Hagen & McPhail, 1970; Bell & Andrews, 1997). Arguing against such a mechanism for stickleback species pairs is the observation that there was a striking clustering of benthic and limnetic sticklebacks within Paxton and Enos lakes (Fig. 3). This implies that the members of each species pair within these lakes shared a common ancestor more recently than either species has with benthics or limnetics in the other lakes or with any solitary lake or marine/anadromous population, a result contrary to the expectations of the double-invasion hypothesis.

For the other species pair lakes, however, our data are less clearly interpreted in terms of the double invasion/sympatric speciation hypotheses. First, in these other cases no other pair was monophyletic (Fig. 3) contrary to predictions of sympatric speciation. Second, in two lakes (Priest and Emily) limnetic sticklebacks showed greater nucleotide divergence from the putative ancestral anadromous sticklebacks, which is inconsistent with the predictions from the double invasion scenario (McPhail, 1993), but in Paxton and Enos lakes the reverse pattern was observed.

Although our data are consistent with a model of sympatric speciation for the Paxton and Enos lakes' species pairs, two alternative explanations are possible. First, within-lake monophyly could be explained by double invasions of each lake by local genetic subpopulations of anadromous or marine sticklebacks that had different genetic characteristics from source colonists near other species pair lakes. Such a

scenario, however, would require highly structured anadromous/marine populations, but our data suggest that this is not so; among population variation in mtDNA accounted for only 3% of the total in anadromous/marine populations, and one haplotype (12) predominated in all anadromous populations (Table 3).

Second, speciation in *Gasterosteus* species pairs may have occurred by double invasions, but hybridization following secondary contact may have obscured ancestral relationships and yielded apparent within-lake monophyly in the Paxton and Enos lakes' pairs. Some first generation hybrids are produced in each lake in nature, but they are rare, usually on the order of less than 1% of morphological samples taken have hybrid-like intermediate morphology (McPhail, 1984, 1992). Further, there is evidence from work in fish and other taxa of mtDNA introgression between hybridizing species (e.g. Tegelström, 1987; Echelle & Conner, 1989; Bernatchez *et al.*, 1995; Gill, 1997; Ruedi, Smith & Patton, 1997) and in Paxton Lake benthics and limnetics have nearly identical mtDNAs (Table 3). The reproductive ecology of the Paxton Lake species pairs suggests that, under experimental conditions, hybridization may be asymmetric and that it most likely occurs between benthic females and limnetic males (Schluter & Nagel, 1995). Transfer of mtDNA between species could be facilitated by backcrossing of female hybrids to males of either parental species. If such matings occurred primarily in the direction of female hybrids bearing benthic mtDNA (i.e. offspring from female benthic × male limnetic matings) with limnetic males, the ancestral affinity of limnetic and anadromous/marine stickleback, predicted by the double invasion hypothesis, would be obscured. Such a scenario, however, depends critically on the microhabitat and mate selection tendencies of female hybrids, both of which are presently unknown.

In summary, our data suggest that in two lakes the species pairs of *Gasterosteus* may provide a further example of genetic evidence consistent with sympatric rather than allopatric speciation as a mechanism of divergence, particularly for fish (Meyer *et al.*, 1990; Taylor & Bentzen, 1993; Bush, 1994; Schliewen *et al.*, 1994; Strecker *et al.*, 1996; Wood & Foote, 1996; Pigeon *et al.*, 1997). Although sympatric speciation may have occurred for some species pairs of sticklebacks, the geography of speciation in the other lakes remains unclear. Even for the Paxton and Enos lakes' pairs, however, we cannot completely discount an alternative scenario of allopatric speciation with mtDNA introgression following secondary contact (double invasions). Corroborating data from nuclear loci may distinguish these alternatives and these ideas are currently being tested by assessing the degree of concordance between mtDNA and nuclear phylogenies using microsatellites (Taylor, 1998).

### *Ecological speciation in sticklebacks*

Our results suggest that sympatric species pairs of *Gasterosteus* have arisen independently in different lakes in south-western British Columbia. In combination with earlier studies of the comparative ecology of these species pairs, our data strongly suggest that these divergences are a result of parallel bouts of selection for specialization in alternative trophic niches. Our data, therefore, strongly implicate natural selection as a major cause of the evolution of the species pairs. Further, the adaptive radiation appears to have proceeded rapidly because the lakes with species pairs have only been accessible to fish for about the last 12 000 years (McPhail & Lindsey, 1986). Notwithstanding the remaining uncertainties over the geography of

the initial divergence, it appears that the evolutionarily significant divergences of both trophic and reproductive phenotypes occurred independently within each lake and in the face of gene flow which was initially either considerable (under a sympatric speciation scenario) or at least moderate (under an allopatric speciation and secondary contact scenario). In both cases, however, selection against hybrids is implicated in the continued maintenance of distinct gene pools in each two species lake (McPhail, 1984, 1992; this study). For instance, in Paxton Lake benthics and limnetics are indistinguishable in terms of mtDNA, yet each species remains distinct in morphology and ecology and shows consistent and significant differences at several allozyme and microsatellite loci (McPhail, 1992; E.B. Taylor, unpubl. data). Given that $F_1$ and $F_2$ hybrids show no reduction in viability or fertility (McPhail, 1984, 1992; Hatfield, 1996) and that there is no evidence of sexual selection against hybrids (Hatfield & Schluter, 1996), there appears to be no hybrid disadvantage based on these life history variables. By contrast, the striking differences in trophic ecology between the parental species, the intermediate trophic morphology, and reduced growth and feeding performance of hybrids in limnetic and benthic habitats (McPhail, 1984, 1992; Bentzen & McPhail, 1984; Schluter, 1993, 1995) all point strongly to an ecological basis for selection against hybrids and the maintenance of genetic integrity in the face of gene flow, and perhaps to the speciation process itself. The combination and integration of phylogenetic and ecological studies of *Gasterosteus* species pairs have, therefore, produced strong evidence for the role of 'ecological speciation' (Schluter, 1996a,b) in adaptive radiation. Ecological speciation highlights the role of divergent natural selection as a mechanism of adaptive radiation, an idea with considerable historical precedent (e.g. Simpson, 1944; Lack, 1947), but comparatively little empirical support in natural populations (Noor, 1995; Schluter, 1996a,b).

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