Effects of colonization history and landscape structure on genetic variation within and among threespine stickleback (*Gasterosteus aculeatus*) populations in a single watershed

Eric J. Caldera¹ and Daniel I. Bolnick²*

¹Department of Zoology and Department of Bacteriology, University of Wisconsin-Madison, 4325 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706 and ²Section of Integrative Biology, University of Texas at Austin, One University Station C0930, Austin, TX 78712, USA

**ABSTRACT**

**Question:** How do post-glacial colonization history and current watershed geomorphology affect population genetic structure in lacustrine fish populations?

**Study system:** Eleven populations of lacustrine threespine sticklebacks (*Gasterosteus aculeatus*) from a single watershed, and two adjacent marine populations of *G. aculeatus* as outgroups.

**Methods:** Individuals were genotyped using six microsatellite loci and several population genetic parameters were calculated (including genetic differentiation, genetic diversity, migration, and divergence time). We regressed population genetic parameters against environmental variables such as stream gradient and length, distance from the mouth of the watershed, and lake area.

**Results:** Genetic differentiation was highest between lakes separated by high-gradient streams, but was unaffected by stream length. Estimated divergence times between adjoining populations declined from >10,000 years ago for lake pairs close to the ocean to less than 5000 years for lake pairs near the top of the watershed. Genetic diversity declined as a function of geographic distance from the mouth of the watershed but was positively correlated with lake area.

**Conclusions:** Our findings confirm expectations that landscape features such as stream gradient and stream branching structure strongly influence patterns of genetic divergence. However, in contrast to previous studies, we found no significant relationship between geographic distance and genetic divergence among lake pairs. We present novel evidence that post-glacial colonization occurred gradually over a significant period of time, rather than a single rapid invasion into all lakes.

**Keywords:** divergence, landscape genetics, microsatellite, migration, population structure.

* Author to whom all correspondence should be addressed. e-mail: danbolnick@mail.utexas.edu
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INTRODUCTION

Many species have patchy distributions across a landscape (Karieva, 1990). Both the evolutionary and ecological dynamics of these metapopulations depend on the rate at which individuals move between patches (Slatkin, 1985; Karieva and Ebert, 2004). Migration rates in turn are heavily influenced by the structure of the abiotic environment (Manel et al., 2003). In freshwater organisms such as fish, migration patterns can be influenced by aspects of watershed structure such as channel slope (Kruse et al., 1997; Rich et al., 2003), flow rates (Armstrong et al., 1997; Petty and Grossman, 2004; Hänfling and Weetman, 2006), altitude (Shaw et al., 1991; Angers et al., 1999; Bohlin et al., 2001; Castric et al., 2001; Poissant et al., 2005), cascades and falls (McGlashan and Hughes, 2000; Costello et al., 2003; Crispo et al., 2006), lake and stream area (Kruse et al., 1997; Dunham and Rieman, 1999), and various anthropogenic disturbances (Angers et al., 1999; Dunham and Rieman, 1999; Wofford et al., 2005; Hänfling and Weetman, 2006; Raeymaekers et al., 2008).

Gene flow (migration that results in the transfer of alleles) tends to homogenize allele frequencies among populations exchanging migrants (Lenormand, 2002). Measures of genetic divergence and diversity can reflect this homogenization, where increased gene flow among populations tends to inhibit divergence among, but increase diversity within, populations. However, genetic structure and diversity can also reflect historic events such as range expansion or colonization (Mayr, 1963; Hewitt, 1996; Grant, 1998). Such genetic signatures are frequently detected from post-glacial expansions in freshwater fish (Angers et al., 1999; Durand et al., 1999; Nesbo et al., 1999; Kotlik and Berrebi, 2001; Hänfling et al., 2002; Volckaert et al., 2002; Gagnon and Angers, 2006). Here, we evaluate the roles of both watershed structure and historical events (post-glacial colonization) on patterns of genetic variation among populations of threespine sticklebacks (Gasterosteus aculeatus).

Study system

Threespine sticklebacks are a predominantly marine species that has colonized freshwater environments following deglaciation in the northern hemisphere. During this colonization, sticklebacks rapidly and repeatedly adapted to diverse freshwater habitats, providing extraordinary cases of phenotypic divergence and parallel evolution (Bell and Foster, 1994; Schluter, 2000; McKinnon and Rundle, 2002). Adaptive phenotypic divergence occurs both within and among lakes (Lavin and McPhail, 1985, 1986; Schluter and McPhail, 1992; Ólafsdóttir et al., 2007a, 2007b), and among marine, lake, and stream sticklebacks (Gross and Anderson, 1984; Bell and Foster, 1994; Deagle et al., 1996; Thompson et al., 1997; Hendry et al., 2002; Hendry and Taylor, 2004; Colosimo et al., 2005; Kitano et al., 2007; Ólafsdóttir et al., 2007b).

Many studies have described patterns of genetic variation in sticklebacks to draw conclusions about current and historical genetic structure (Haglund et al., 1992; Ortí et al., 1994; Deagle et al., 1996; Higuchi and Goto, 1996; Higuchi et al., 1996; Taylor and McPhail, 1999; Reusch et al., 2001; Watanabe et al., 2003; Johnson and Taylor, 2004; Raeymaekers et al., 2005, 2007, 2008; Mäkinen et al., 2006; Malhi et al., 2006; Kitano et al., 2007). Many of these studies evaluated the racemic model of evolutionary diversification in sticklebacks (Williams, 1992), which posits that marine populations represent a large evolutionarily stable stem lineage that gives rise to large numbers of small side-branches in the form of variable but extinction-prone freshwater populations (Bell, 1976; Bell and Foster, 1994). Studies using hyper-variable markers such as microsatellites have examined among-habitat and among-watershed genetic structure, lending support to the racemic model. Other studies have evaluated the relative roles of habitat versus geography in structuring genetic variation.
For instance, Mäkinen et al. (2006) conducted a large-scale study of threespine sticklebacks across Europe and found evidence for genetic similarity based on geographic region, but not clustering by habitat. Similarly, a study conducted in Western Europe found evidence of drainage playing a larger role in structuring populations than habitat (Raeymaekers et al., 2005). At a finer spatial scale, Reusch et al. (2001) examined populations of threespine sticklebacks from lakes, rivers, and estuaries in Germany and found evidence for genetic clustering along habitat lines. Overall, the results of these studies suggest that both geography and adaptation to different habitats influence patterns of genetic variation. However, relatively few studies have evaluated the roles of geography and history at the finer scale of individual watersheds. Raeymaekers et al. (2008) provide a rare example, showing that anthropogenic changes in river flow affect patterns of genetic divergence.

Landscape genetics (Manel et al., 2003) within a watershed is of particular interest, because this is the spatial scale at which migration is most likely to influence adaptive divergence among populations (Hendry et al., 2002; Moore and Hendry, 2005; Kitano et al., 2007; Moore et al., 2007; Bolnick et al., 2008).

In this paper, we evaluate how abiotic watershed features have influenced patterns of genetic diversity and divergence in a metapopulation of lake-dwelling threespine sticklebacks. In particular, we evaluate the effects of stream characteristics on migration between adjoining lakes, of lake size on genetic diversity, and of the branching structure of the watershed on patterns of genetic similarity. We also infer effects of colonization history and use divergence time estimates to test whether there were significant lag times during colonization of the lower versus upper regions of the watershed, or whether colonization occurred as a single rapid invasion.

**METHODS**

**Sampling and genotyping**

We used minnow traps to collect a total of 598 threespine sticklebacks (*Gasterosteus aculeatus*) from 11 freshwater lakes within the Amor de Cosmos watershed, and from two additional nearby marine populations on Vancouver Island, British Columbia, Canada (Fig. 1; sample sizes and geographic data in Table 1). The Amor de Cosmos watershed was not available for colonization until approximately 12,000 years B.P., when glacial retreat uncovered the landscape and isostatic rebound lifted present-day low-elevation regions up from below sea level (Hagen and McPhail, 1970; Bell and Foster, 1994; Clague and James, 2002). Some low-lying lakes in the region may have been subject to a second period of submergence during a localized brief sea level rise of ∼50 m, dated to about 1500–2000 years after deglaciation (Taylor and McPhail, 2000; Clague and James, 2002; Johnson and Taylor, 2004). All of the lakes in the Amor de Cosmos watershed are more than 50 m above present-day sea level, so were not affected by this secondary submergence. Several lakes in the watershed lack stickleback populations, apparently because the outlet river gradients are too steep to allow fish to disperse upstream to these sites.

Fin clips of captured fish were placed in ethanol and stored at −20°C until DNA was extracted with Qiagen DNAeasy tissue kits. Each fish was genotyped at six di-repeating uninterrupted microsatellite loci belonging to separate linkage groups: stn9, stn130, stn171, stn177, stn195, and stn207 (Peichel et al., 2001). We followed the PCR mixture and temperature profile of Peichel et al. (2001), using FAM reverse-labelled primers. Microsatellite repeat lengths were visualized by running 1 µl of PCR product on a 16 capillary ABI 3130 with
GENESCAN-500 (ROX) as an internal size standard, and alleles were scored using GeneMarker® 1.3. Lastly, we employed the program MICRO-CHECKER (van Oosterhout et al., 2004) to examine possible null alleles and errors in scoring.

Analyses

We used GENEPOP on the web (Raymond and Rousset, 1995) to conduct several standard population genetic analyses, including tests for deviations from Hardy-Weinberg and linkage equilibrium, and estimates of $F_{IS}$ according to Weir and Cockerham (1984). Locus- and population-level deviations from Hardy-Weinberg equilibrium and linkage equilibrium were calculated using Fisher’s method of combining test results (Manly, 1985).

We used ARLEQUIN (Excoffier et al., 2005) to perform an AMOVA with $R_{ST}$ (sum of squared size differences) based distances, to test for significant among-population variation. ARLEQUIN also provided pairwise $R_{ST}$ and Slatkin’s linearized $F_{ST}$ values between all populations (Slatkin, 1995). To assess the statistical significance of $F_{ST}$ values, the null distribution of pairwise $F_{ST}$ values is first generated by permuting haplotypes between populations, with the assumption of no difference. The $P$-value is the proportion of permutations leading to an $F_{ST}$ larger or equal to the observed one.

Fig. 1. Map of the Amor de Cosmos watershed on Northern Vancouver Island, British Columbia, Canada. The lower left box displays the entire island with labels on the two sampled marine populations and the Amor de Cosmos watershed. The upper right-hand box is an enlarged image of the Amor de Cosmos watershed with the 11 sampled freshwater lakes labelled.
Table 1. Basic information about sampled populations' geography and genetic diversity

<table>
<thead>
<tr>
<th>Lake</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Sample size</th>
<th>Allelic diversity</th>
<th>Genetic diversity (π)</th>
<th>Elevation (m)</th>
<th>Area (ha)</th>
<th>Max. depth (m)</th>
<th>Mean depth (m)</th>
<th>Distance from ocean (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campbell River Estuary (CRE)</td>
<td>50°2'50&quot;N</td>
<td>125°15'37&quot;W</td>
<td>29</td>
<td>59</td>
<td>4.982</td>
<td>0</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Sayward Estuary (SWE)</td>
<td>50°22'46&quot;N</td>
<td>125°56'43&quot;W</td>
<td>30</td>
<td>67</td>
<td>4.994</td>
<td>0</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>McCreight (MC)</td>
<td>50°17'7&quot;N</td>
<td>125°38'53&quot;W</td>
<td>29</td>
<td>66</td>
<td>4.863</td>
<td>60</td>
<td>272</td>
<td>55</td>
<td>31</td>
<td>6 363</td>
</tr>
<tr>
<td>Farewell (FW)</td>
<td>50°12'5&quot;N</td>
<td>125°35'9&quot;W</td>
<td>50</td>
<td>67</td>
<td>4.702</td>
<td>180</td>
<td>18.75</td>
<td>12</td>
<td>5</td>
<td>23 676</td>
</tr>
<tr>
<td>Muskeg (MK)</td>
<td>50°12'5&quot;N</td>
<td>125°34'29&quot;W</td>
<td>50</td>
<td>47</td>
<td>3.444</td>
<td>188</td>
<td>12.18</td>
<td>3</td>
<td>2</td>
<td>24 680</td>
</tr>
<tr>
<td>Cedar (CD)</td>
<td>50°12'13&quot;N</td>
<td>125°34'3&quot;W</td>
<td>50</td>
<td>31</td>
<td>2.767</td>
<td>204</td>
<td>31</td>
<td>20</td>
<td>3</td>
<td>25 875</td>
</tr>
<tr>
<td>Blackwater (BW)</td>
<td>50°10'39&quot;N</td>
<td>125°35'20&quot;W</td>
<td>50</td>
<td>41</td>
<td>4.434</td>
<td>188</td>
<td>37.5</td>
<td>28</td>
<td>15</td>
<td>26 257</td>
</tr>
<tr>
<td>Amor (AM)</td>
<td>50°9'27&quot;N</td>
<td>125°34'42&quot;W</td>
<td>45</td>
<td>54</td>
<td>4.53</td>
<td>209</td>
<td>362.21</td>
<td>49</td>
<td>15</td>
<td>30 149</td>
</tr>
<tr>
<td>Ormond (OR)</td>
<td>50°10'51&quot;N</td>
<td>125°31'30&quot;W</td>
<td>51</td>
<td>23</td>
<td>2.273</td>
<td>227</td>
<td>14.1</td>
<td>20</td>
<td>10</td>
<td>34 073</td>
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<tr>
<td>Dugout (DO)</td>
<td>50°11'0&quot;N</td>
<td>125°31'28&quot;W</td>
<td>60</td>
<td>21</td>
<td>2.238</td>
<td>227</td>
<td>3.5</td>
<td>2</td>
<td>1</td>
<td>34 404</td>
</tr>
<tr>
<td>Roberts (RO)</td>
<td>50°12'55&quot;N</td>
<td>125°32'29&quot;W</td>
<td>54</td>
<td>60</td>
<td>4.488</td>
<td>160</td>
<td>161.3</td>
<td>53</td>
<td>25</td>
<td>22 782</td>
</tr>
<tr>
<td>Little Mud (LM)</td>
<td>50°12'24&quot;N</td>
<td>125°33'3&quot;W</td>
<td>50</td>
<td>29</td>
<td>2.798</td>
<td>205</td>
<td>4.29</td>
<td>12</td>
<td>5</td>
<td>35 493</td>
</tr>
<tr>
<td>Mud (MU)</td>
<td>50°12'6&quot;N</td>
<td>125°33'3&quot;W</td>
<td>50</td>
<td>27</td>
<td>2.946</td>
<td>208</td>
<td>38.73</td>
<td>23</td>
<td>7</td>
<td>33 467</td>
</tr>
</tbody>
</table>

*Note:* Allelic diversity is the number of alleles sampled in each lake and π (pairwise differences) is an absolute measure of genetic diversity.
We next used the Bayesian program STRUCTURE (Pritchard et al., 2000) to determine the number and identity of genetically distinguishable clusters. To calculate the most probable number of clusters, we ran a model allowing for between $K = 1$ to $K = 13$ clusters, with each $K$ iterated 10 times, omitting information on individuals’ sampling locations. In addition to calculating posterior probabilities associated with each $K$, we used a $\Delta K$ statistic according to Evanno et al. (2005) to select the optimal $K$. All STRUCTURE analyses were run with a burn-in of 100,000 and a run time of 300,000.

Having found significant genetic divergence among populations, our next goal was to determine whether watershed structure influenced the pattern of divergence. In particular, if geographically closer populations tend to be more similar (due to more recent common ancestry or current gene flow), we would expect patterns of divergence to reflect the branching pattern of the watershed. We used genetic distances to construct a cluster diagram of population relationships using PHYLIP 3.65 (Felsenstein, 2005). We estimated a consensus tree using Cavalli-Sforza chord distances, $D_c$ (Cavalli-Sforza and Edwards, 1967), and the neighbour-joining (NJ) method. $D_c$ distances assume drift is more important than mutation, allow for variable population sizes, and have proved effective for microsatellite analyses of population structure (Takazaki and Nei, 1996; Beebee and Rowe, 2000). To assess the stability of nodes, 1000 bootstrap replications were performed. Within the PHYLIP package, the programs SEQBOOT, GENDIST, and NEIGHBOR were used to create replica data sets, calculate $D_c$, and construct NJ trees respectively. The final consensus tree was produced in CONSENSE, employing the majority rule method.

Next, we tested several hypotheses about how watershed geomorphology influenced observed levels of genetic divergence between adjacent population pairs (directly connected by a stream). We obtained measures of river length and lake elevations from the Freshwater Fisheries Society of British Colombia (www.fishwizard.com). Multiple regression tested whether the level of divergence between pairs of populations (linearized $F_{ST}$) depends on the length of the stream connecting two lake populations and/or on the mean gradient of the stream (elevation change/distance). In cases where variables did not conform to a normal distribution (by a Shapiro-Wilk normality test) and transformations could not normalize the data, we used non-parametric Spearman rank correlations. Two sets of lake pairs are not entirely independent: Farewell/McCreight and Roberts/McCreight share a short stream segment in common. Because the shared stream segment is short relative to their separate stream beds ($\approx 17\%$), we treat them as independent pairs. We omitted a contrast between Roberts and Farewell, which would be redundant because of each lake’s contrast with McCreight. Similarly, the two comparisons of McCreight Lake with the two estuary populations traverse a single stream segment, so we averaged their $F_{ST}$ values to obtain a single data point for that stream.

To determine whether river currents bias the direction of any migration, we estimated reciprocal migration rates (upstream and downstream) between adjacent populations with BAYESASS 1.3 (Wilson and Rannala, 2003), using default parameters with an increased run time of 10,000,000 generations. Output from BAYESASS is interpreted as the proportion of individuals in the recipient population inferred to be recent immigrants from the specified source population. Because BAYESASS 1.3 focuses on contemporary migration rates (the past two generations), estimates are unaffected by the colonization process and are thus appropriate for detecting upstream/downstream biases. Paired $t$-tests were used to evaluate whether downstream migration was consistently higher than upstream migration, paired by stream segment. We then regressed mean reciprocal migration rates against stream length.
connecting two lake populations and stream gradient. Unlike $F_{ST}$, migration rates appeared to have a non-linear relationship with stream gradient, which we evaluated using the nlm function in R to test for a logistic (threshold) function.

Although sticklebacks are known to have invaded freshwater habitats such as the Amor de Cosmos watershed from marine populations, the progression of such colonization is uncertain. In particular, was watershed colonization effectively instantaneous, or did it occur in a gradual stepping-stone fashion with significant lags between successive upstream colonizations? We addressed this question in several ways. First, we tested for stronger bottlenecks in populations further upstream. The mean number of pairwise differences ($\pi$, estimated in ARLEQUIN) and estimates of effective population size ($N_e$; from IM, described below) in each population were regressed against the lake’s distance from the ocean (following the riverbed), as well as lake size (surface area). If upstream populations exhibit reduced genetic diversity, we would infer that upstream movement is rare enough that significant lag times are likely. As a further test for historical bottlenecks (within the past 0.2 to $4N_e$ generations), we used the program BOTTLENECK (Piry et al., 1999) to test for elevated heterozygosity relative to the number of alleles at each locus under a stepwise mutation model (SMM).

In a second test of sequential colonization timing, we used the results of the Bayesian population genetic program IM (Hey and Nielsen, 2004) to estimate the divergence time between adjoining populations, their effective population sizes, and reciprocal migration rates. IM assumes that an ancestral population split into two populations that subsequently exchange migrants, and estimates how long ago that split occurred ($t \mu$). We ran IM for every pairwise combination of adjoining lakes. Each run of IM continued until ESS values for each parameter reached >50, using a burn-in of 1,000,000 generations, constant population sizes, and five simultaneous chains with a two-step heating scheme. Because IM performs poorly when no significant genetic differences exist, we only report results from lake pairs with significant $F_{ST}$ values. IM failed to converge when applied to lake pairs with non-significant $F_{ST}$ values, yielding highly variable estimates in replicate runs and low ESS values (<50) even after >80 million cycles.

IM’s parameter estimates for divergence time ($t \mu$) and effective population sizes ($4N_e \mu$) are scaled by the mutation rate, $\mu$. To convert these scaled estimates into an estimate of the number of generations (or individuals), we estimated the mean microsatellite mutation rate using a calibration corresponding to the end of glacial coverage. Based on the geological history of the region, we assumed that McCreight Lake was first colonized from marine populations shortly after deglaciation, hence $t = 12,000$ years ago (Hagen and McPhail, 1970; Clague and James, 2002). Using IM, the scaled divergence time between McCreight Lake stickleback and marine populations (averaging IM runs for McCreight vs. Sayward or Campbell River) was $t = 0.505$. The two estimates using different marine populations were similar. Using a 1-year generation time [sticklebacks in these lakes mature within a year, and most die before their second year (D.I. Bolnick unpublished data)], we infer a microsatellite mutation rate $\mu = 4.21 \times 10^{-5}$, which falls within the range of published values for dinucleotide repeats (Bachtrog et al., 2000; Vazquez et al., 2000; Dupuy et al., 2004). We then used this mutation rate to convert the parameter estimates for other lake pairs into absolute estimates of $t$ or $N_e$. We regressed $t$ between adjacent lake pairs against the upper lake’s distance from the ocean. A significant negative slope would be consistent with a lag time during watershed colonization, with lakes further up the watershed being colonized significantly later. Note that error in our mutation rate estimate alters the intercept but not the slope of this regression. We also regressed $N_e$
against lake size and distance from the ocean. As some lakes were used in multiple analyses of different lake pairs, we averaged the estimates of effective population size (N_e) obtained for a given population across IM runs (Table 2).

RESULTS

Population genetic structure and gene flow

Five of the six microsatellite loci amplified with a success rate above 99%, the exception being locus stn9 (success rate 90%). Individuals with non-amplifying stn9 alleles were restricted to four lakes, in close proximity to each other (Farewell 14%, Blackwater 30%, Muskeg 10%, Cedar 67%) (Fig. 1). Individuals with failed amplifications for stn9 consistently worked for all other loci, indicating that the extracted DNA was intact. In an attempt to successfully genotype the stn9 locus, we re-designed multiple primer pairs at different points in the flanking region surrounding the stn9 microsatellite repeat, but the re-designed primers also failed to amplify these individuals. We posit that these null alleles represent a sizeable insertion or deletion in the region of the microsatellite locus. Thus, for the purpose of genetic analysis, we treated individuals with non-amplifying stn9 as homozygotes for a recessive null. In lakes where these null individuals are found, it is likely that some individuals scored as homozygotes for other stn9 alleles may in fact be heterozygotes. This error rate is likely to be fairly low (for instance, this affects at most four individuals in Cedar Lake) and does not affect our subsequent analyses, which are qualitatively similar with or without stn9.

Three populations deviated from Hardy-Weinberg equilibrium: Little Mud, Dugout, and Ormond. Relatively small estimates of effective population size (N_e) and genetic diversity in these lakes (Tables 1 and 2) may contribute to these deviations via the force of genetic drift. There is some evidence of assortative mating in lacustrine sticklebacks (Snowberg and Bolnick, submitted), which may also contribute to deviations in Hardy-Weinberg equilibrium. Two loci showed whole-watershed deviations from Hardy-Weinberg equilibrium (stn207 and stn177) but the occurrence across lakes was random. Analysis with MICRO-CHECKER found examples of heterozygote excess and deficiency that may have been caused by scoring errors due to stutter and null alleles respectively; however, these examples caused no significant deviations in Hardy-Weinberg equilibrium. Finally, we found no evidence for linkage disequilibrium between any pairs of loci.

An AMOVA confirmed that there is substantial genetic structure within the Amor de Cosmos River watershed, with 26% of genetic variation attributed to differences between populations (P < 0.0001), with among-population variation ranging from 15% to 43% for individual loci (P < 0.0001). Supporting the AMOVA results, Bayesian simulations conducted in STRUCTURE grouped individuals to into nine genetic clusters based on the calculation of posterior probabilities (Fig. 2). Individuals were generally assigned to clusters that corresponded to individual lakes. The exceptions are four clusters representing pairs of adjacent populations (Farewell and Black Water; Ormond and Dugout; Little Mud and Mud; Sayward and Campbell River estuaries). The first three of these pairs are closely adjoining lakes with very similar elevation and negligible FST. The latter pair of populations are marine populations that are 73 km apart.

In contrast to the nine clusters identified based on posterior probabilities, the ΔK method of Evanno et al. (2005) yielded K = 4 populations (Fig. 2). These groups were composed of
Table 2. Results of Bayesian simulations performed in IM

<table>
<thead>
<tr>
<th>Population</th>
<th>Assumption: $\mu = 0.000042$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Downstream</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>CRE</td>
<td>MC</td>
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<td>SWE</td>
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</table>

Note: The first two columns contain adjacent pairwise populations followed by scaled estimates of effective population size for each pair of lakes, the effective population size of the hypothetical ancestral population of the lake pair, divergence time of the lake pair in generations, and the upstream and downstream migration rates between the lake pairs. $m_{ds}$ is interpreted as migration from the downstream population into the upstream population, while $m_{us}$ is interpreted as migration from the upstream population into the downstream population. The 95% CI follow each parameter estimate. Population pairs with grey rows did not yield repeatable results in replicate IM results and failed to converge to high ESS values even after >100,000,000 MCMC generations, presumably due to insufficient genetic differences between populations. For population abbreviations, see Table 1.
Ormond and Dugout, Mud and Little Mud, Muskeg and Cedar, and then all other lakes together with the marine populations. Intermediate-elevation lakes (Farewell, Blackwater, and Amor) were placed primarily in the latter group, with some admixture from the high-elevation pairs. Simulations by Evanno et al. (2005) suggest that when there is hierarchical genetic structuring, their $\Delta K$ statistic detects the highest level of genetic structure, so finding only four groups with this method does not contradict the finer-scale divergence reflected in the $K = 9$ outcome.

Patterns of genetic similarity among populations based on a neighbour-joining tree using Cavalli-Sforza chord distances also largely mirrored watershed topology (Fig. 3). The two marine populations clustered together, and were used to root the tree of freshwater populations. Overall, five phylogenetic clades were consistent with the topology of the watershed. Only two discrepancies were noted with watershed branching patterns: (1) a cluster comprised of Mud and Little Mud was placed as a sister clade to the Farewell/Blackwater/Muskeg/Cedar group, rather than to Roberts Lake into which they drain (Fig. 3); and (2) Amor, Ormond, and Dugout Lakes formed a cluster that was placed as sister to the Mud/Farewell/Blackwater clade, even though they drain into Blackwater Lake, and hence should be nested within that clade. In both cases of mismatch between watershed and genetic topology, the stream segment in question contains a substantial stretch of high-gradient flow lacking sticklebacks and thus represents a major barrier to gene flow.

Pairwise $F_{ST}$ and $R_{ST}$ values are presented in Table 3. There was a significant correlation between the topographic slope between lakes and pairwise $F_{ST}$ values between adjacent lakes ($r_s = 0.863, P = 0.001$; Fig. 4A). In contrast, the distance separating lake pairs had no significant association with pairwise $F_{ST}$ ($r_s = -0.091, P = 0.797$), and the trend was not in the direction expected if geographic distance poses a barrier to migration (Fig. 4B). Multiple regression confirmed the results of these non-parametric tests, but assumptions of normal residuals were violated.

Analyses using BAYESASS did not yield any evidence of consistent up- or down-stream migration bias ($t_{11} = -0.39, P = 0.698$). Two lines of evidence suggested that these migration estimates using BAYESASS are biologically meaningful. First, although BAYESASS provided non-zero migration rates for all population pairs regardless of geographic proximity, adjoining lakes had generally higher migration estimates than non-adjacent lakes ($t = 2.15, P = 0.012$). Second, consistent with results noted above for $F_{ST}$, mean migration
Fig. 3. A comparison of watershed topology with the topology of microsatellite genetic distances. The cladogram of population similarity is presented in panel (A). Tree topology is estimated from Cavalli-Sforza chord distance ($D_c$), and bootstrap support greater than 50% is reported at relevant nodes. Panel (B) illustrates the branching order of the watershed, but is not to scale or north–south orientation. Shading highlights five different phylogenetic clades that are consistent with the topology of the watershed. Connecting streams with large stretches of stickleback-free habitat (due to high-gradient flow) are marked with asterisks.

Table 3. Genetic divergence ($F_{ST}$ upper right, based on allele frequencies; $R_{ST}$ lower left, based on microsatellite allele frequencies and sizes) among 11 freshwater lakes and two marine populations (Sayward and Campbell River), using six microsatellite loci

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* Non-significant genetic divergence ($P > 0.05$); all other values are significant. For population abbreviations, see Table 1.
rate along a stream segment was negatively correlated with stream gradient ($r_S = -0.727, P = 0.015$; Fig. 5) and unrelated to stream length ($r_S = -0.339, P = 0.280$). Multiple regression found similar results (gradient $P = 0.023$, distance $P = 0.178$). There appears to be an appreciable non-linear effect of stream gradient on mean reciprocal migration rates (Fig 5), which was confirmed by significant logistic regression driven by what appears to be a threshold stream gradient (asymptote: $t = 2.715, P = 0.024$; inflection: $t = 2.881, P = 0.018$), below which migration rates are high enough to homogenize populations. This threshold effect is driven by four population pairs with very high migration estimates with BAYESASS (Fig. 5). These population pairs (Farewell and Blackwater Lakes, Mud and
Little Mud, and Sayward and Campbell River estuaries) have negligible \( F_{ST} \) values, short low-gradient connecting streams, and were not separated by STRUCTURE even with \( K = 9 \). When genetic divergence is so weak, BAYESASS performs poorly. Indeed, for these lakes the posterior probability confidence intervals were similar to what is expected from uninformative data (Wilson and Rannala, 2003), and the migration rate estimates were massively asymmetrical. Consequently, we consider the four high-migration points in Fig. 5 to be very imprecise estimates. Removing these four points, we still found a strong (and linear) effect of stream gradient on migration rates for the higher-gradient streams (\( r_S = -0.773, P = 0.008 \); Fig. 5), with no sign of an asymptote.

**Genetic diversity and colonization history**

The two marine populations (Sayward and Campbell River) yielded the highest values for \( \pi \) (mean number of pairwise differences, a measure of genetic diversity), followed by McCreight (the closest lake to the mouth of the watershed) (Table 1, Fig. 1). There was a negative correlation between \( \pi \) for a given lake and its distance from the mouth of the watershed (\( r_S = -0.815, P = 0.004 \); Fig. 6A). There was also a positive relationship between \( \pi \) and lake surface area (\( r_S = 0.69, P = 0.040 \); Fig. 6B). We detected no significant non-
linearity. We detected no significant relationship between lake size and watershed distance \( (r_S = -0.30, P = 0.437) \), but when we used multiple regression to test for both variables’ effects on \( \pi \), only the distance effect was significant (distance \( P = 0.009 \), area \( P = 0.100 \)). Consequently, the surface area effect might be spurious.

The negative association between diversity and distance from the ocean suggests that stickleback populations experienced successive reductions in effective population size in the process of colonizing the watershed. Estimates of effective population size (\( N_e \)) calculated with IM (Table 2) were also negatively associated with distance from the mouth of the watershed \( (r_S = -0.88, P = 0.001; \text{Fig. 6C}) \) and positively associated with lake surface area \( (r_S = 0.74, P = 0.024; \text{Fig. 6D}) \). These correlations are consistent with the trends in genetic diversity. In multiple regression, the distance effect is significant \( (P = 0.002) \) but the area effect is not \( (P = 0.149) \). The results also support the utility of IM in returning biologically reasonable inferences, as IM’s estimates of effective population size were obtained independently of any information on watershed location or lake size. We also obtained estimates of effective population size and migration rates using MIGRATE (Beerli and

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**Fig. 6.** Effect of lake characteristics (distance from the ocean and size) on genetic diversity \( \pi \) (mean pairwise differences), and effective population sizes inferred from Bayesian analyses with IM.
However, it would appear that our parameter estimates obtained with MIGRATE were unreliable: effective population size estimates were unrelated to lake size or distance from the ocean, and migration rates were uncorrelated with stream distance or gradient. When we used an unconstrained migration matrix, migration rates were not significantly higher between adjoining lakes than between lakes on opposite ends of the watershed. MIGRATE may perform poorly with low numbers of loci (Abdo et al., 2004), so we do not present these results in detail.

Despite the clear trend for reduced effective population size further up the watershed, we found no evidence for recent bottlenecks. Analyses with the program BOTTLENECK found no significant excess of heterozygosity given the number of alleles. Two hypotheses could explain the disagreement between the BOTTLENECK results and the genetic diversity data. First, the bottlenecks occurring during colonization could have been of a magnitude that escapes detection by BOTTLENECK while still leaving an across-lake trend in diversity. Alternatively, the bottlenecks could have been strong but restricted to the initial colonization events, allowing sufficient time for heterozygosity to recover to its equilibrium expectation. In principle, BOTTLENECK can detect bottlenecks as old as \(4N_e\) generations ago, which should encompass the initial colonization events of these lakes. Interestingly, Hänfling and Weetman (2006) also found a pattern of declining genetic diversity in sculpin (Cottus gobio) moving up a watershed, yet BOTTLENECK did not detect any signature of drastic reductions in \(N_e\).

To determine the timing of colonization, we used IM to estimate divergence times (\(t\)) between adjacent populations. We found that divergence times between adjoining lake pairs decreased as a function of the lake pair’s distance from the mouth of the watershed (\(r_S = -0.786, P = 0.048\); Fig. 7). The exact number of generations depends linearly on our assumed mutation rate, but the trend for more recent divergence higher in the watershed remains significant regardless of which mutation rate we use to scale the actual values. The

![Fig. 7.](image_url)

**Fig. 7.** The divergence time estimates (\(t\), in years) for pairs of adjacent lakes as a function of the distance from the watershed mouth to the lake. Divergence times are obtained by assuming a microsatellite mutation rate of \(\mu = 4.2 \times 10^{-5}\), which places the McCreight Lake split with marine populations at 12,000 years ago. Assuming other mutation rates changes the scale of the y-axis, but not the trend across lake pairs.
watershed was deglaciated approximately 12,000 years B.P., giving a calibration point for divergence between McCreight and marine populations. The oldest divergence time estimate (16,310 years B.P.) is between McCreight and Roberts Lake. While this estimate exceeds the post-glacial origin of the watershed, its 95% confidence interval includes the more geologically realistic value of 12,000 years B.P. (Table 2). In contrast, the most recent divergence time estimate occurred between Muskeg Lake and Cedar Lake, estimated at 2024 years ago. The implication is that the watershed was colonized gradually over thousands of years, rather than rapidly in one brief period of colonization.

**DISCUSSION**

Genetic variation among populations likely reflects a combination of historical events and ongoing processes such as gene flow. When evaluating these historical and contemporary population genetic patterns, a researcher’s conclusions may depend on the spatial scale being examined. Most previous studies of stickleback population genetics have ranged from global scales to surveys of continent-wide phylogeography or variation among watersheds (Haglund et al., 1992; Ortí et al., 1994; Deagle et al., 1996; Higuchi and Goto, 1996; Higuchi et al., 1996; Taylor and McPhail, 1999; Reusch et al., 2001; Watanabe et al., 2003; Johnson and Taylor, 2004; Raeymaekers et al., 2005, 2007; Mäkinen et al., 2006; Malhi et al., 2006). We are aware of only one study that focuses specifically on patterns of genetic variation among numerous stickleback populations within a single watershed (Raeymaekers et al., 2008), which concluded that anthropogenic changes to water flow affected population genetic structure. A few additional studies have examined parapatric divergence between pairs of populations (Hendry et al., 2002; Hendry and Taylor, 2004; Bolnick et al., 2008) to evaluate the effects of migration–selection balance. Many other studies have examined genetic variation at the watershed scale in other fish taxa, and have found evidence for genetic structure arising from watershed geomorphology [e.g. Trinidad guppies *Poecilia reticulata* (Crispo et al., 2006); river sculpin *Cottus gobio* (Hanfling and Weetman, 2006); Dolly Varden char *Salvelinus malma* (Koizumi et al., 2006)].

We conducted a whole-watershed analysis of lacustrine threespine sticklebacks, testing for both historical and contemporary influences on population genetic variation. Consistent with previous studies of freshwater fish (Shaw et al., 1991; Angers et al., 1999; Hanfling et al., 2002; Costello et al., 2003; Crispo et al., 2006; Raeymaekers et al., 2008), we find that both colonization history and ongoing gene flow contribute to current patterns of genetic structure. Our study also provides novel insights into the nature of the colonization process. In particular, it appears that post-glacial colonization into freshwater habitats was not rapid, but rather occurred slowly over thousands of years.

**Colonization history**

The racemic model of stickleback evolution posits that marine populations represent a large evolutionarily stable stem lineage, which gives rise to large numbers of small and extinction-prone side branches in freshwater environments (Bell, 1976; Bell and Foster, 1994). A fundamental prediction of the racemic model is that freshwater populations should be less genetically diverse than marine populations. Low measures of genetic diversity in freshwater versus marine sticklebacks have been detected previously (Rafinski et al., 1989; Taylor and McPhail, 1999, 2000; Reusch et al., 2001; Raeymaekers et al., 2005; Mäkinen et al., 2006), and were confirmed by our present study. More specifically, by focusing on a single side-branch of the racemic tree, we observed
a successive decline in diversity as one moves from lakes closer to the ocean, further upriver to higher elevation lakes (Fig. 6A, B). This declining diversity has previously been documented in sticklebacks (Malhi et al., 2006; Raeymaekers et al., 2008) and other freshwater species (Shaw et al., 1991; Angers et al., 1999; Costello et al., 2003).

Two scenarios (not mutually exclusive) may contribute to the pattern of decreased genetic diversity with increasing distance upriver from the ocean. First, during initial colonization, stickleback populations may have experienced successive founder effects as a small number of individuals moved upstream to establish populations in empty lakes, losing genetic variation along the way. This signal of colonization history may subsequently decay as mutation, gene flow, and selection alter the genetic structure arising from colonization (Templeton et al., 1995; Latta and Mitton, 1999; Mateoq et al., 2000; Walton et al., 2000). In the second scenario, all colonizing populations may have suffered similar levels of reduced genetic diversity, but subsequent gene flow from marine populations (or upstream sources) subsidized the genetic diversity in low-elevation lakes. In freshwater sticklebacks, previous workers have invoked both successive bottlenecks during colonization (Taylor and McPhail 1999, 2000) and ongoing immigration of marine fish (Johnson and Taylor, 2004; Malhi et al., 2006).

In the Amor de Cosmos watershed, it is likely that the observed upstream decline in genetic diversity is largely due to historical colonization events. The river draining from McCreight into the ocean has at least one large cascade approximately 3 km downstream of the lake that poses a very substantial barrier to upstream dispersal of marine sticklebacks into the lowest-elevation lake of the watershed. McCreight Lake was presumably colonized during post-glacial isostatic rebound or the brief period of sea level rise, when the cascades would have been below sea level, and then received little or no subsequent immigration. Also, we observe decreases in genetic variation even at the highest reaches of the watershed, for example between Amor Lake and Ormond and Dugout, where recurrent marine immigration is likely to be negligible. Downstream subsidies of genetic diversity from high-to low-elevation lakes also appear unlikely, given our finding of no downstream dispersal bias. Consequently, we infer that variation in genetic diversity within the watershed reflects differences in past or present effective population size, rather than genetic subsidies from post-colonization marine immigrants or subsidies from upstream populations. As a result, lakes cluster according to their riverine connection patterns even when the streams in question are steep enough to reduce migration drastically. Cedar and Muskeg Lakes, and Amor and Ormond Lakes, both cluster together despite very little ongoing gene flow, suggesting that their similarity reflects colonization history rather than extensive subsequent migration. The one piece of evidence arguing for genetic subsidies from marine populations is that we found low $F_{ST}$ values between McCreight Lake and the two marine populations. Based on the major dispersal barrier downstream of McCreight, we posit that this genetic similarity reflects the legacy of recent common ancestry and large population sizes, rather than migration rates under migration–drift equilibrium.

A second major finding regarding colonization history is that the invasion of the Amor watershed was likely a protracted process. Population pairs closer to the ocean were inferred to have split from a common ancestor at a much older date than pairs higher up the watershed (Fig. 7). Using an initial colonization date of 12,000 years to calibrate the genetic inferences, we conclude that the highest-elevation lakes were colonized nearly 10,000 years after the initial invasion began. Such lag times suggest that river currents were sufficient to pose substantial barriers to upstream dispersal, delaying colonization of upstream lakes. In the rare event of successful colonization, one would expect a significant bottleneck.
Consequently, the colonization lag times dovetail with the pattern of reduced genetic diversity, both of which suggest that colonization was a rare event. Several lakes in the watershed still lack sticklebacks, due to steep intervening streams.

**Landscape impacts on genetic structure and diversity**

The isolating nature of river systems typically translates to greater genetic divergence among freshwater populations than among marine populations (Waples, 1998; DeWoody and Avise, 2000). In our study, one of the lowest levels of genetic divergence was found between the two marine populations (Campbell River Estuary and Sayward Estuary: $F_{ST} = 0.01; R_{ST} = 0.012$; Table 2), despite being geographically much further from each other than any two lake populations surveyed. The greater geographic structure among freshwater populations presumably reflects low rates of migration between lakes. What mechanisms lead to reduced migration? Some recent microsatellite studies of freshwater sticklebacks have documented isolation by distance (Reusch et al., 2001; Raeymaekers et al., 2005, 2008). In contrast, we found no significant effect of stream length on divergence between adjoining lakes. Instead, we found that genetic divergence between adjoining lakes is positively related to the gradient of the intervening stream. This result is consistent with the few other studies to test for effects of stream steepness. In a study of guppies, Crispo et al. (2006) observed a positive relationship between genetic divergence and the number of waterfalls between populations. Similarly, Lowe et al. (2006) provide strong evidence for isolation by slope in a freshwater stream salamander.

Sticklebacks are known to establish permanent populations in freshwater streams that can be a conduit for migration between lakes. Stream populations have been documented in the Amor watershed (Hendry and Taylor, 2004), and mark–recapture studies have found some individuals that have dispersed between lakes [upstream from Little Mud into Mud Lake (D.I. Bolnick, unpublished results)]. However, even short stretches of high-gradient streams may present substantial barriers. A few streams, such as those connecting Roberts and Little Mud Lake, or Blackwater to Amor Lake, are consistently steep enough that they contain long stretches with no resident sticklebacks. The presence of stream sticklebacks might explain why isolation by distance plays less of a role in genetic structure within this watershed than physical barriers to migration imposed by high-gradient stretches of stream.

If steep stream gradients and/or cascades and waterfalls pose substantial barriers to upstream dispersal, it is reasonable to assume that any movement across these barriers would be biased in the downstream direction, regardless of the fish species. We therefore expected to observe a downstream bias in the proportion of migrants supplied to an adjacent lake [as has been found in sculpin (Hannfng and Weetman, 2006)]. However, reciprocal migration rates from BAYESASS do not support any downstream or upstream bias. One possible explanation for this finding is the observation that lake sticklebacks tend to swim upstream when placed in streams (Hendry et al., 2002). This tendency towards upstream movement (rheotaxis) may counteract the ease of downstream versus upstream movement over barriers.

The existence of stream sticklebacks is a complicating factor that we wish to incorporate into future analyses (for an analysis of connectivity in riverine sticklebacks, see Raeymaekers et al., 2008). Previous studies have shown that gene flow from lake populations may oppose local adaptation in stream sticklebacks (Hendry et al., 2002; Hendry and Taylor, 2004; Moore and Hendry, 2005; Moore et al., 2007). Conversely, habitat differences between lake and stream sites may result in significant
natural selection against lake fish venturing into streams. Selection is frequently strong enough to create morphological clines from lake into stream populations despite high levels of gene flow (D. Berner, personal communication). Natural selection could thus exaggerate the strength of barriers between lakes, as well as maintaining genetically divergent populations in close proximity to lake populations. Habitat differences between lakes and streams could also lead to reduced migration via adaptive habitat choice. If lake fish avoid entering stream habitats (and vice versa), streams may offer little opportunity for between-lake gene flow (D.I. Bolnick, unpublished results). Such habitat preferences may be weak initially during colonization by marine fish, but become more pronounced as the sticklebacks adapt to their lake or stream habitats.

In sticklebacks, previous studies of molecular genetic variation have ranged from holarctic phylogeography, to continent-wide variation, down to the scale of among and within watersheds, and divergence between parapatric habitats. We provide a fine-scale look at genetic variation within a single watershed, focusing on 11 lake populations of sticklebacks plus two marine populations. Our results confirm expectations that landscape features such as stream gradient and stream branching structure strongly influence patterns of genetic divergence. Furthermore, we present evidence that colonization occurred via a gradual stepping-stone process over a significant period of time, rather than a single rapid invasion into all lakes. These inferences complement the larger-scale studies of among-watershed and among-continent divergence, and provide a foundation for future studies of adaptive divergence among populations within a watershed. Watershed-level studies of population genetics and migration are particularly important, as this is the spatial scale at which migration–selection balance and other micro-evolutionary processes are most relevant.

ACKNOWLEDGEMENTS

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