doi:10.1111/j.1558-5646.2009.00665.x

VARIABLE PROGRESS TOWARD ECOLOGICAL SPECIATION IN PARAPATRY: STICKLEBACK ACROSS EIGHT LAKE-STREAM TRANSITIONS

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Received September 26, 2008
Accepted February 2, 2009

Divergent selection between contrasting habitats can sometimes drive adaptive divergence and the evolution of reproductive isolation in the face of initially high gene flow. "Progress" along this ecological speciation pathway can range from minimal divergence to full speciation. We examine this variation for threespine stickleback fish that evolved independently across eight lake-stream habitat transitions. By quantifying stickleback diets, we show that lake-stream transitions usually coincide with limnetic-benthic ecotones. By measuring genetically based phenotypes, we show that these ecotones often generate adaptive divergence in foraging morphology. By analyzing neutral genetic markers (microsatellites), we show that adaptive divergence is often associated with the presence of two populations maintaining at least partial reproductive isolation in parapatry. Coalescent-based simulations further suggest that these populations have diverged with gene flow within a few thousand generations, although we cannot rule out the possibility of phases of allopatric divergence. Finally, we find striking variation among the eight lake-stream transitions in progress toward ecological speciation. This variation allows us to hypothesize that progress is generally promoted by strong divergent selection and limited dispersal across the habitat transitions. Our study thus makes a case for ecological speciation in a parapatric context, while also highlighting variation in the outcome.

KEY WORDS: Adaptive divergence, foraging morphology, *Gasterosteus aculeatus*, gene flow, genetic differentiation, reproductive isolation.

Theoretical work suggests that speciation can occur despite initially high gene flow. This "divergence with gene flow" context for speciation can occur in parapatry (Endler 1977; Gavrilets et al. 2000; Doebeli and Dieckmann 2003; Gavrilets 2004), or in sympatry (review: Bolnick and Fitzpatrick 2007). The possibility of sympatric speciation has received most of the recent attention, and a number of high-profile papers have recently argued for its presence in particular natural systems (Barluenga et al. 2006; Savolainen et al. 2006; Ryan et al. 2007; Hunt et al. 2008). Parapatric speciation, however, is predicted to be much more common than sympatric speciation, both from a theoretical perspective, and based on the ubiquity of spatially structured populations and ecological transitions in nature (Schilthuizen 2000; Gavrilets 2000, 2004; Coyne and Orr 2004). Empirical evidence for parapatric speciation nevertheless remains thin (for strong candidates see Grahame et al. 2006; Panova et al. 2006; Foster et al. 2007; Quesada et al. 2007; Seehausen et al. 2008). The main empirical challenge is that an alternative speciation scenario—divergence in isolation followed by hybridization after secondary contact—can result in similar spatial structuring and genetic signatures, and is therefore notoriously difficult to rule out (Barton and Hewitt 1985; Jiggins and Mallet 2000; Coyne and Orr 2004).

A key condition promoting parapatric speciation is the occurrence of ecological transitions in space (ecotones) (Schilthuizen 2000; Gavrilets 2004). Ecotones can generate strong divergent selection and allow some adaptive divergence in space even if

gene flow is initially substantial (Endler 1977; Hendry et al. 2001; Gavrilets 2004; Gavrilets and Vose 2005; Thibert-Plante and Hendry 2008). This partial adaptive divergence should then begin to restrict gene flow owing to reproductive barriers caused by, for example, ecological selection against migrants and hybrids, the evolution of habitat preferences, or mate choice linked to the traits under selection (Schluter 2001; Coyne and Orr 2004; Nosil et al. 2005; Rundle and Nosil 2005). This initial restriction on gene flow should then allow further adaptive divergence, which should generate further ecologically based reproductive barriers (Rice and Hostert 1993). The outcome of this feedback process can range from weak population differentiation all the way to fully reproductively isolated, genetically discontinuous clusters, as well as other possible intermediate states (Wu 2001; Dres and Mallet 2002; Mallet 2008; Thibert-Plante and Hendry 2008). The factors determining how far along this speciation continuum diverging populations will progress remain little explored. The challenge here is that multiple replicate instances of ecological divergence need to be compared. We begin such an investigation by examining divergent selection, adaptive divergence, and gene flow across multiple replicate habitat transitions in nature.

Threespine stickleback fish (*Gasterosteus aculeatus*) can often be found across transitions between lake and stream habitats. Divergent selection between these habitats should be substantial because lakes and streams are very different in several physical and biotic features. Most importantly, lake habitats typically provide substantial limnetic (pelagic) prey whereas stream habitats provide almost exclusively benthic prey, and this ecological contrast has been inferred to mediate divergent selection driving phenotypic divergence (Berner et al. 2008). The opportunity for gene flow across lake-stream transitions should also be high because the two habitats occur in direct contact, generally without significant physical barriers to dispersal. Indeed, gene flow can sometimes constrain adaptive divergence between lake and stream habitats (Hendry et al. 2002; Hendry and Taylor 2004; Moore et al. 2007). The opposite process—adaptive divergence constraining gene flow (i.e., ecological speciation)—has not been formally examined for lake-stream stickleback. We here combine estimates of divergent selection with phenotypic and neutral marker data to test for signatures of ecological speciation in parapatric lake-stream stickleback. We further make use of the availability of multiple replicate lake-stream transitions to identify variation in progress toward speciation, and to explore possible determinants of progress.

Our analysis starts with a quantification of stickleback resource use across eight separate lake-outlet stream transitions. We predict downstream shifts in foraging from somewhat limnetic to primarily benthic, which would suggest that lake-stream transitions represent real ecotones mediating divergent selection (details below). We then examine foraging morphology across the transitions, specifically testing for spatial associations between shifts in resource use and shifts in morphology. Such an association would imply that morphological change reflects ecologically based adaptive divergence. We next use microsatellite marker data to confirm that stickleback in the eight lake-stream transitions can indeed be considered evolutionarily independent replicates, and to examine genetic population structure across the transitions. The finding of substantial differentiation in neutral genetic markers, linked to shifts in resource use and foraging morphology, would imply that divergent selection has driven the evolution of generalized barriers to gene flow (Rundle and Nosil 2005; Räsänen and Hendry 2008; Thibert-Plante and Hendry 2008). Finally, we use the marker data to explore whether divergence could have occurred in the face of initial gene flow, i.e., in parapatry.

Materials and Methods

STICKLEBACK SAMPLES

Our study is based on stickleback collected from eight lake-outlet stream "systems" on Vancouver Island, British Columbia, Canada. Within each system, fish were sampled from the lake in immediate proximity (0–30 m distance) to the lake-outlet stream transition and from six sites along the outlet stream, resulting in seven clinal samples per system and 56 samples in total. Geographic coordinates for all sites are given in Table S1. We attempted to sample along each stream at intervals of approximately 500 m. However, due to limited accessibility, unsuitable habitat, or short outlet streams, the distance between adjacent sample sites ultimately varied from 110 m to 1533 m (mean = 511 m, SD = 310 m). The maximum distance between the lake site and the farthest stream sites varied from 1540 to 4390 m among the lake-stream systems.

The stickleback used for morphological and genetic analyses were captured with unbaited minnow traps or dip nets in the spring of 2006, except for the Misty system, where comparable collections had been made in the spring of 2003. Within each lake-stream system, all of the samples were collected within a few days of each other. Of the fish captured at each site, we haphazardly selected and retained 20 individuals, excluding those less than a year old (estimated from their size) and those showing any signs of gravidity. The retained fish were killed with an overdose of MS-222 and immediately photographed with a digital camera. For the photographs, each fish was placed left-side-up in a natural position on a standard background with a reference scale. Fine pins were used to highlight landmarks otherwise difficult to locate on the photograph. The fish were subsequently transferred to 95% ethanol.

The above samples were not processed in a way that allowed the optimal preservation of stomach contents. We therefore collected an additional 20 fish at each of the same 56 sites in the spring of 2007. The sampling protocol was the same as above,

except that the traps were always inspected less than 4.5 h after they were set. We then killed the fish immediately, opened their abdominal cavity, and transferred them to ethanol. We assume that stomach content data from the 2007 fish is representative of previous years because we sampled at identical locations and at the same time in the season in both years. Temporal consistency in diet has been confirmed for other stickleback populations in one of our study watersheds (Bolnick et al. 2008), although fine-scale variation has been seen in another system (Reimchen and Nosil 2002).

SURROGATE FOR DIVERGENT SELECTION

Previous work on lake-stream stickleback used water flow and depth measurements to infer divergent selection between lakes and streams, while acknowledging the need for a better surrogate (Moore et al. 2007). Here we take up this challenge by using information on limnetic vs. benthic prey types found in the stomachs of stickleback. The assumption that diet data reflect selection on foraging traits is supported by the strong correlation between foraging morphology and limnetic versus benthic diets within and among stickleback populations (Gross and Anderson 1984; Schluter and McPhail 1992; Robinson 2000; Berner et al. 2008). Furthermore, the functional link between foraging morphology and foraging performance (i.e., limnetic vs. benthic) has been confirmed by laboratory and field experiments (Bentzen and McPhail 1984; Schluter 1993, 1995; Robinson 2000). In addition, we have shown directly for stickleback in the Misty system that individuals with a limnetic-adapted morphology display limnetic stomach contents when sampled from the lake, but benthic stomach contents when sampled from the outlet stream (Berner et al. 2008). This suggests that foraging tracks local resource availability rather than being strongly determined by the phenotype. All of these results suggest that diet differences provide a reasonable surrogate for divergent selection on foraging-related morphology in stickleback.

Prey items retrieved from stickleback stomachs were identified under a stereomicroscope at 15–45× magnification and classified as limnetic (open water), benthic (in or on the substrate), or "other" (potentially in the open water or on the substrate). Identification and classification was based on criteria given in Pennak (1989) and Thorp and Covich (2001). Limnetic prey included mainly nonchydorid cladocera, calanoid copepods, and emerging mayflies and diptera. Benthic prey included mainly diptera larvae (Chironomidae and Ceratopogonidae), chydorid cladocera, mayfly and caddis fly larvae, and ostracoda. "Other" prey included primarily cyclopoid copepods. Following previous work (Schluter and McPhail 1992; Berner et al. 2008), we calculated the proportion of limnetic prey to limnetic and benthic prey combined (hereafter "proportion of limnetic prey," or "PLP"), and used this metric for analysis. For 30 haphazardly selected individuals, we recounted and reclassified prey items on a later occasion, and then used these data to estimate the repeatability (Lessells and Boag 1987) of PLP. Repeatability was reasonably high ($r = 0.80$; likely an underestimate because some prey items were lost during handling and preservation of stomach content after the first inspection).

FORAGING MORPHOLOGY

Our study focuses on two key phenotypic variables: body shape and gill raker number. These two variables are clearly under divergent selection between limnetic (zooplankton) and benthic (macro-invertebrate) foraging modes. In particular, previous studies have shown that stickleback with shallower (more streamlined) bodies and more numerous gill rakers feed primarily and more efficiently on zooplankton, whereas those with deeper bodies and fewer gill rakers feed primarily and more efficiently on macroinvertebrates (Bentzen and McPhail 1984; Gross and Anderson 1984; Schluter and McPhail 1992; Schluter 1995; Robinson 2000; Bolnick 2004). A strong genetic basis has generally been demonstrated for population differences both in body depth (Lavin and McPhail 1993; Robinson 2000; Hendry et al. 2002; Schluter et al. 2004; Albert et al. 2008; Sharpe et al. 2008) and gill raker number (Gross and Anderson 1984; Lavin and McPhail 1993; Hatfield 1997; Peichel et al. 2001; Robinson 2000; Hendry et al. 2002), although direct evidence is lacking for the specific systems studied here. Moreover, lake-stream divergence in these traits has been shown to be reasonably consistent across different years (Hendry et al. 2002; Hendry and Taylor 2004; Moore et al. 2007).

We quantified body shape divergence with geometric morphometrics, where lake-stream divergence has been found to be genetically based (Sharpe et al. 2008). We digitized 17 landmarks on each photograph using tpsDig software (Rohlf 2001).The landmark configuration (Fig. 1A) was modified from Walker (1997) in that we excluded the caudal tip of the posterior process of the pelvic girdle, as well as the posterior tip of the ectocoracoid. Instead, we included the posterior edge of the eye, the base of the first pectoral fin ray, and the base of the pelvic spine. We also introduced a slider (semilandmark) anterior to the first dorsal spine landmark. This slider was defined by the intersection of the body outline and the line perpendicular to the snout-tail axis at the posterior edge of the operculum. The resulting landmark dataset was subjected to analysis in tpsRelw (Rohlf 2001), thus obtaining individual centroid size, affine (uniform components), and nonaffine (partial warps) attributes of shape variation (Zelditch 2004), as well as their principal components (relative warps, RWs).

To obtain gill raker number, specimens were dissected after approximately 3 months of preservation. The total number of gill rakers on the first left branchial arch was then counted under a stereomicroscope at 15× magnification, and sex was determined. Gill raker number was analyzed as untransformed counts.

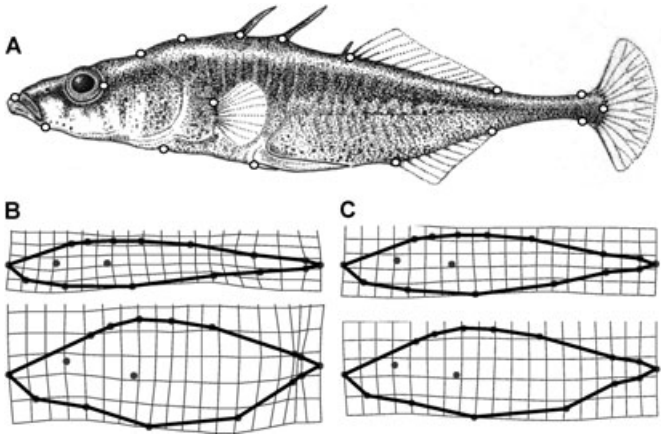

Figure 1. (A) Landmark configuration used to quantify body shape in lake-stream stickleback (see text for details). (B) First principal component (relative warp) of shape variation among all pooled individuals (total $N = 1129$). This major axis of shape variation captures primarily changes in body depth along the entire body axis and in the length of the caudal peduncle. (C) Body shape change along the first canonical variate for the distance term in the global GLM reveals a similar pattern. (B) and (C) display the individuals with the highest and lowest observed scores for first relative warp and first canonical variate.

Error arising during data acquisition was assessed by counting the rakers and digitizing the landmarks (from the same photographs) on two separate occasions for 24 haphazardly selected individuals. Measurement error was minimal, as indicated by the high repeatability for RWs 1–3 (all $r > 0.991$) and gill raker number ($r = 0.918$).

MICROSATELLITES AND POPULATION GENETIC ANALYSIS

DNA was extracted from stickleback pectoral fin tissue using QIAGEN DNeasy kits (Qiagen Inc., Valencia, CA) following the manufacturer's protocols. Six dinucleotide microsatellite markers were then amplified by polymerase chain reaction (conditions as specified in Peichel et al. 2001) using fluorescent phosphoramide labels. The specific loci were chosen so as to be unlinked to each other and to any known quantitative trait locus (QTL) (Peichel et al. 2001; Colosimo et al. 2004): Stn67 (linkage group 6), Stn159 (13), Stn238 (4), Stn171 (15), Stn195 (20), and Stn207 (25). The products were run on an ABI 3730 sequencer (Applied Biosystems Inc., Foster City, CA) and analyzed using GENEMAPPER version 3.7 (Applied Biosystems) at Génome Québec (Montreal, Canada). The same two individuals and two blinds were included on each plate to confirm consistency in allele sizes and the absence of contamination.

We tested each sample-locus combination for deviations from linkage and Hardy–Weinberg equilibrium in GENEPOP version 4 (Rousset 2008). For each locus, we examined the resulting *P*-values both before and after sequential Bonferroni correction for multiple testing ($\alpha = 0.05$, $K = 56$) (Rice 1989). We also calculated the binomial probability for the observed frequency of significant tests (e.g., Hendry and Taylor 2004). For descriptive purposes, we then calculated F_{ST} (Weir and Cockerham 1984) for all sample site pairings within each watershed using GENETIX version 4.05 (Belkhir et al. 2004). We also recoded the marker data with RECODEDATA version 0.1 (Meirmans 2006) to recalculate F_{ST} values standardized by average heterozygosity (Hedrick 2005). These standardized values also range from 0 to 1 and give the proportion of the maximum differentiation possible for a given level of genetic variation within populations.

To assess evolutionary independence of our systems, we used the microsatellite data to evaluate genetic relationships among our samples. We here used UPGMA with Nei's (1972) standard genetic distance, as implemented in POPULATIONS version 1.2 (Langella 2002). The use of different distance metrics and cluster algorithms, as well as the exclusion of single loci produced very similar results (not presented). Among these additional methods was the maximum likelihood method available in the ContML package of PHYLIP version 3.67 (Felsenstein 2004). We further note that genetic relationships among our samples likely reflect patterns consistent across years, as this has been shown for several lake-stream populations (Hendry et al. 2002; Hendry and Taylor 2004).

To examine whether lake-stream stickleback within systems formed genetically differentiated populations, we used STRUCTURE version 2.1 (Pritchard et al. 2000). This Bayesian clustering program identifies distinct populations by minimizing linkage and Hardy–Weinberg disequilibrium. We chose the admixture and independent allele model with 100,000 repetitions as burnin and run lengths. (The correlated allele option and only 10,000 repetitions for burnin and runs produced highly consistent results—not shown). Five replicate simulations for $K = 1$–4 (the number of populations assumed) were performed. We then averaged log-likelihoods across the five runs and determined the most likely value for K in each watershed by applying the ΔK method (Evanno et al. 2005). Because this method cannot identify situations in which most probable $K = 1$, the results were examined further following recommendations provided in the STRUCTURE version 2.2 documentation (Pritchard and Wen 2004).

The STRUCTURE analysis indicated that two genetically distinct populations occurred in six of the eight lake-stream systems (see Results). We explored whether these populations had diverged in the face of gene flow by using IMa (Hey and Nielsen 2007). This program provides a Bayesian coalescent-based implementation of a general model in which an ancestral population splits into two populations that may exchange genes in both directions at unequal rates during divergence. Preliminary simulations allowed specifying appropriate priors for each system and showed

that robust results were obtained with search strategies that included 35–66 Metropolis-coupled Markov chains with geometric heating (parameters 0.95 and 0.9) and chain length of 4.5–10 million genealogies following burnin (1 million). Five replicate runs with different random number seeds were performed for each system. We used the program to obtain estimates of unidirectional migration rates between the two populations (m_1, m_2), and of the time since splitting (t). We present these estimates scaled by the mutation rate as given directly by IMa. In addition, we converted m to the effective number of immigrants per generation by using IMa's estimates of theta (scaled effective population size), and converted t to the approximate number of generations. For the latter, we assumed a mutation rate per locus and generation of 10^{-4}, a reasonable value for dinucleotide repeats in fish (e.g., Yue et al. 2007; Caldera and Bolnick 2008).

Because nonzero gene flow between populations can indicate either divergence with gene flow or hybridization in secondary contact following differentiation in allopatry (Barton and Hewitt 1985; Jiggins and Mallet 2000), we also examined the number and distribution of migration events along the IMa simulations for each locus. This procedure was used to obtain qualitative insights into the alternative possibilities of divergence with gene flow (migration events distributed broadly over the course of simulation) versus recent hybridization following secondary contact (concentration of migration events near the present) (Won and Hey 2005; Niemiller et al. 2008).

We note here that the standard coalescent model, as implemented in IMa, assumes that populations are panmictic, of constant size during divergence, and more closely related to each other than to other (unsampled) populations (Kingman 1982). Although the last of these condition is almost certainly satisfied in our stickleback systems, some departures from panmixia and constant population size are inevitable, which may have influenced model parameter estimation (Wakeley 2000). Furthermore, it is unknown how reliably divergence history is recovered when using a relatively modest number of microsatellite markers, here six. These limitations warrant some caution in the interpretation of the simulation results.

STATISTICAL ANALYSIS

We first tested for spatial changes in selective conditions by using univariate general linear models (GLMs) with the proportion of limmentic prey (PLP) as response variable, lake-stream system as factor, geographical distance from the lake as covariate, and the system by distance interaction.

We next explored spatial shifts in foraging morphology. For body shape, we used two different approaches. In the first, we analyzed all RWs capturing more than 10% of the total shape variation in separate univariate GLMs. These models included system as factor, distance from the lake within each system and centroid size as covariates, and all interactions. Second, we analyzed shape in a multivariate GLM (MANCOVA) with affine and nonaffine variation combined as response matrix, and model terms as in the previous analysis. Gill raker number was analyzed in a GLM with system as factor, distance as covariate, and the interaction.

To test for an association between foraging morphology and local foraging conditions, we first calculated residuals of individual RW1 scores regressed against centroid size. These size-adjusted RW1 scores, gill raker number, and PLP were then averaged within each sample site. (Working with site means instead of individuals as datapoints was necessary here because morphological and PLP data were from different individuals). Finally, we analyzed site means for RW1 and gill raker number in separate GLMs with system as factor, mean PLP as covariate, and the interaction.

Because of significant lake-stream system by distance interactions, all the above GLMs were also performed within each system separately (hereafter referred to as "system-specific" as opposed to "global" analyses). We additionally ran all above analyses separately for males and females, which produced highly consistent results (details not shown). We therefore present only results from analyses with the sexes pooled.

As mentioned earlier, STRUCTURE identified two genetically distinct populations in six systems. We here tested whether the genetic clusters were also differentiated in average prey resource use. The rationale behind this test was that a consistent association between local selective conditions and neutral genetic differentiation provides strong indication of ecologically based barriers to gene flow (Räsänen and Hendry 2008). This association was tested within each of the six systems by taking a randomization approach. For this, we first aligned and averaged individual population assignment probabilities over the replicate STRUCTURE simulations by using CLUMPP version 1.1.1 (Jakobsson and Rosenberg 2007). We then calculated for each sample site the average probability of belonging to the inferred genetic cluster containing the lake site. Based on the average probability and using 0.5 as cut-off, sample sites were then grouped together to form the genetic "upstream" (including the lake site) and "downstream" clusters. We then calculated average PLP values for the two clusters of sites, retained the absolute difference as a test statistic, and evaluated the significance of its observed magnitude against a random distribution. This distribution was created by shuffling individual PLP values between the upstream and downstream cluster 9999 times, and for each iteration recalculating the difference in cluster mean PLP. This randomization procedure tested the null hypothesis that an observed association between genetic clustering and foraging mode within a given system was due to chance. This randomization test (and all GLMs above) were performed in R 2.7.0 (R Development Core Team 2008).

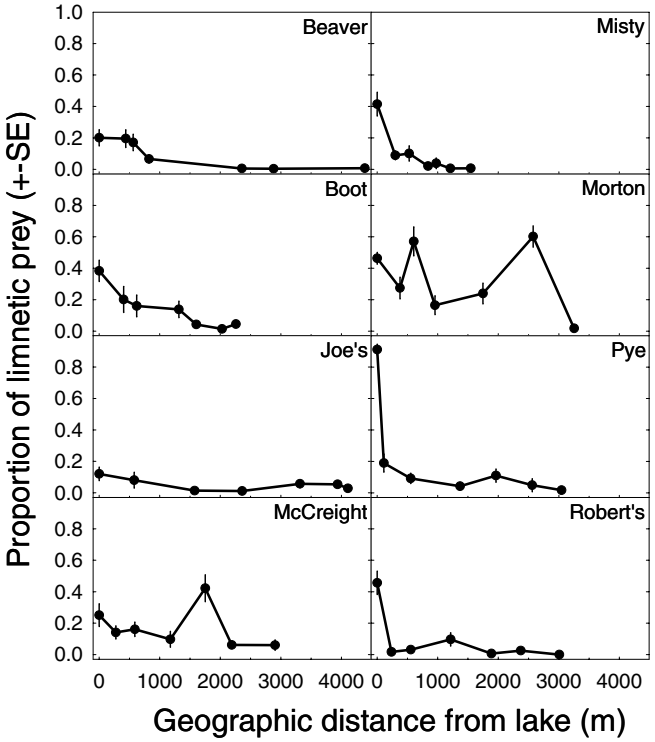

Figure 2. Change in prey types consumed by stickleback across eight lake-outlet stream transitions. The proportion of limnetic prey is typically highest in lake fish and approaches zero downstream.

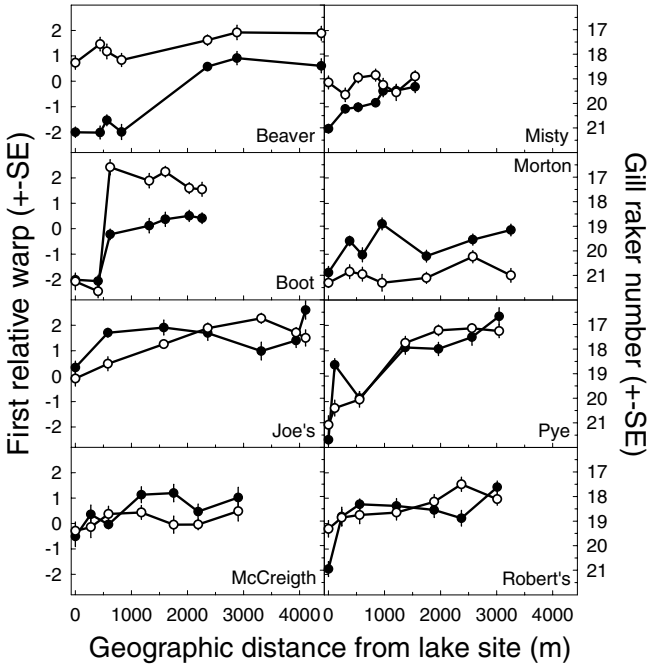

Figure 3. Change in stickleback body shape and gill raker number along eight lake-outlet stream systems. Stream fish generally tend toward deeper bodies (high values for the first relative warp, black dots and left axis), and fewer gill rakers (white dots, right axis). Note that the gill raker axis is inverted.

Results

LOCAL RESOURCE USE

The stomach content analysis largely supported our expectation of changes in food resources across lake-stream transitions. Lake stickleback generally consumed a substantial proportion of limnetic prey (mean 40%, range: 12–91%) (Fig. 2). PLP then usually declined downstream (global model, distance effect: $P < 0.0001$; statistical details given in Table S2), with stream fish typically consuming almost exclusively benthic prey at some distance from the lake. Lake-stream transitions thus usually represent limnetic–benthic ecotones. Notable exceptions were the McCreight and Morton systems in which substantial feeding on limnetic prey also occurred at some stream sites. This variation certainly contributed to the significant system by distance interaction ($P < 0.0001$), indicating that the slopes of spatial change in PLP differed among systems. The effect of distance on PLP was also significant ($P < 0.0195$) in all system-specific tests except McCreight ($P = 0.157$) and (perhaps) Joe's ($P = 0.062$) (details not presented).

SPATIAL SHIFTS IN FORAGING MORPHOLOGY

The major axis of body shape differences among individuals (first relative warp, RW1) explained 25% of the total shape variation and was associated with changes in body depth along the entire body and in the length of the caudal peduncle (Fig. 1B). RW1 showed a clear spatial pattern: stickleback were generally most streamlined in the lake and tended toward greater body depth and shorter peduncles downstream (Fig. 3). This trend was significant in the global GLM (distance: $P < 0.0001$; Table S2), as well as in the system-specific tests (distance: all $P < 0.0026$, details not presented). The global model also indicated that the systems differed substantially in the slope of change in RW1 (system by distance interaction: $P < 0.0001$). RW2 and RW4 (19% and 11% of the variation) mainly reflected body bending and hence variation associated with the position of specimens during photography. RW3 (12% of the variation) reflected individual differences in the length of the base of dorsal and anal fins. These latter three RWs showed no obvious association with distance from the lake (statistical details not presented) and are therefore not considered further. The alternative (multivariate) analysis yielded highly consistent results (global model, distance: $P < 0.0001$, system by distance interaction: $P < 0.0001$; Table S2). Notably, shape variation associated with the first canonical variate for the distance term in the global GLM resembled body shape change along RW1 (Fig. 1C). In short, body shape variation reflecting lake-stream divergence was well captured by RW1.

A general spatial trend was also evident for gill raker number, which usually declined with distance downstream from the lake

(Fig. 3) (global model, distance: $P < 0.0001$; Table S2). The slope of this decline again differed among systems (interaction: $P < 0.0001$). The distance term was also significant ($P < 0.0004$) in system-specific analyses, except for McCreight, Misty, and Morton ($P > 0.11$).

GENETICS

We found no indication of linkage disequilibrium among our microsatellite markers. The number of significant disequilibria was expected by chance ($P = 0.91$), and none survived Bonferroni correction. This result agrees with the known position of our markers on different linkage groups. The markers also only rarely showed significant heterozygote deficiency (before Bonferroni: 2–7 of the 56 samples for a given locus). The observed frequency of significant tests was not higher than expected by chance ($P > 0.145$) for all loci except Stn195 and Stn207 ($P = 0.021$). Only a single test for Stn195 remained significant after Bonferroni corrections. Further, the deviations were distributed relatively evenly among the samples. The few departures from Hardy–Weinberg equilibrium are thus very unlikely to materially influence the following results.

Our stickleback samples were usually more genetically similar within than among systems, except for McCreight and Pye, where the differences were so large within systems that they did not cluster together (Fig. S1). Critically, however, populations from different systems never clustered together closely. Moreover, the two outlet systems (McCreight and Robert's) that we sampled within the same watershed (Amor de Cosmos) were also very genetically distinct and can therefore also be considered independent (a conclusion also reached by Caldera and Bolnick 2008). The maximum-likelihood analysis produced qualitatively similar results (not shown), and so we treat all eight of our systems as effectively independent in an evolutionary sense.

The clustering analysis using STRUCTURE revealed the presence of two genetically differentiated stickleback groups in all systems except for Misty and Morton (Fig. 4). (F_{ST}-based measures of pairwise genetic differentiation among all sample sites within watersheds are presented in Table S3). More specifically, the ΔK method always identified two clusters as most likely. Within the Misty and Morton system, however, group assignment probabilities were essentially $1/K$ for all individuals, a clear indication that stickleback within these two systems formed a single homogenous population ($K = 1$) (Pritchard and Wen 2004). Generally, STRUCTURE produced highly consistent results across replicate simulations. For instance, the among-replicate similarity indices H computed by CLUMPP for the systems with $K = 2$ were all very close to the maximum of 1 (0.973–0.999) (Jakobsson and Rosenberg 2007).

Our simulations with IMa to explore whether genetic clusters within systems might have originated in the face of gene flow were highly consistent across replicates and generally produced clear posterior probability peaks for the model parameters. In all systems, downstream gene flow was clearly nonzero and sometimes (Joe's, Robert's) reached very high values (Fig. 5, Table 1). Upstream gene flow was typically substantially weaker, and it was essentially zero for Beaver, Boot, McCreight, and Pye. The simulations also suggested that divergence was relatively recent: splitting time estimates ranged from approximately a hundred to a few thousand generations (Table 1). Migration events were generally distributed broadly over the estimated divergence period, even though a tendency to concentrate in the second half of the period was evident (Fig. 6). This latter result does not allow us to discriminate between divergence entirely in parapatry versus divergence with a mixture of parapatric and allopatric phases (e.g., due to temporary dispersal barriers in the stream).

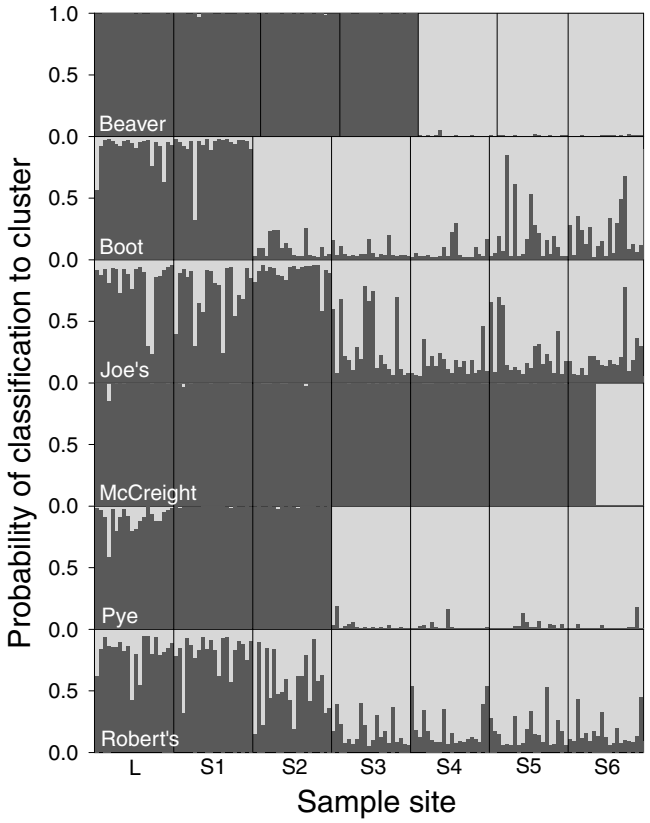

Figure 4. Individual assignment to the two populations (shown in dark and light gray) inferred by STRUCTURE. Individuals are grouped by sample site (L = lake, S1–S6 = stream sites ordered by distance from the lake). The Misty and Morton systems are excluded because there was no population structure.

ASSOCIATIONS BETWEEN RESOURCE USE, MORPHOLOGY, AND POPULATION STRUCTURE

Foraging morphology was clearly related to local prey resource use (Fig. 7): PLP had a highly significant effect on both body shape (RW1, size-adjusted; $P < 0.0001$) and gill raker number

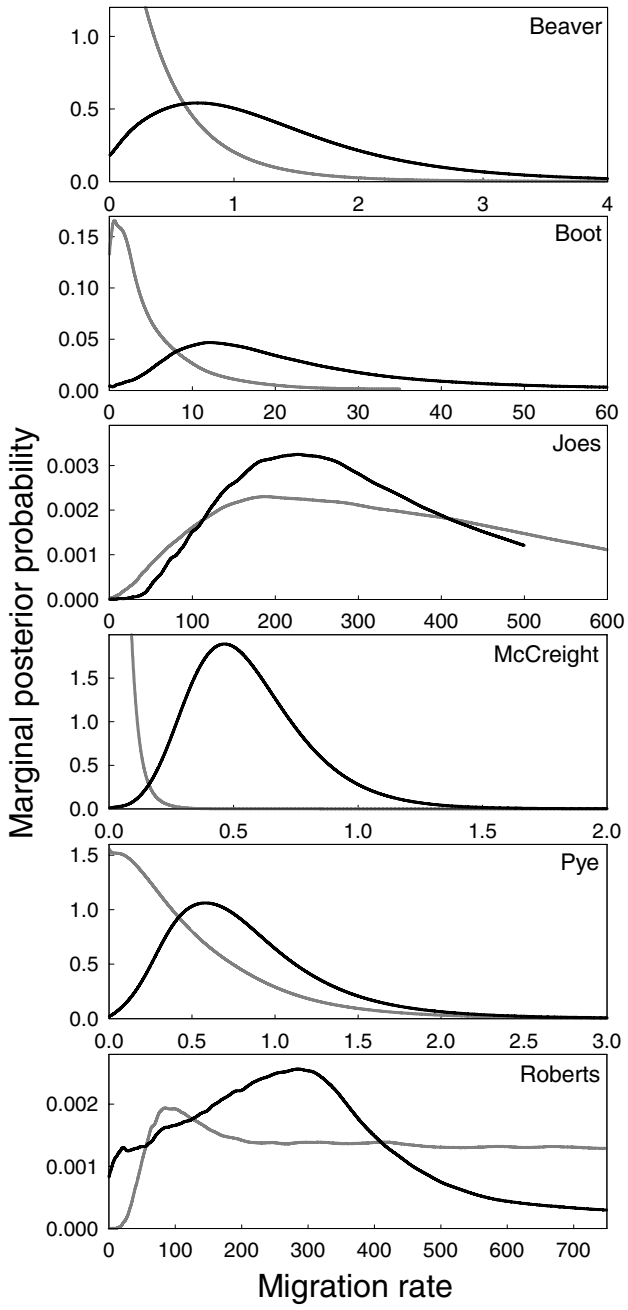

Figure 5. Posterior probability density distributions for unidirectional migration rates (scaled by the mutation rate) as calculated by IMa. Gray and black curves show upstream (m_1) and downstream (m_2) migration, respectively, averaged over five replicate simulations. Nonzero to very substantial downstream (and sometimes upstream) migration rates are indicated. Note that scales vary among the systems. Estimated probability peaks are given in Table 1.

($P = 0.0002$) in the global analysis (details in Table S4). System-specific correlations between RW1 and PLP (details not presented) were consistently in the expected direction (negative), and significantly so (all $P < 0.038$), except for McCreight and Morton ($P > 0.3$). The system-specific association between gill raker number and PLP reached or approached significance only for Boot, Joe's, and Pye ($P < 0.08$; all other systems $P > 0.1$), but was nevertheless in the expected direction (negative) in all but two systems (note that sample sizes were small, $N = 7$ per system). Our randomization tests further made clear that prey resource use also predicted affiliation to the genetically distinct clusters identified by STRUCTURE (Table 2, Fig 7). The observed values for PLP were always higher in the upstream genetic cluster that included the lake fish.

Because the available number of samples within systems did not allow for a formal comparison of the spatial position of breaks in selective conditions, morphology, and genetic clusters, we explored these relationships qualitatively. Most notably, within Joe's, Pye and Robert's, some stream sites in which stickleback genetically classified together with the lake fish were actually more similar to downstream fish in resource use and morphology (Fig. 7). This downstream displacement of genetic breaks relative to shifts in diet and morphology suggests that neutral genetic divergence was sometimes constrained by ongoing gene flow.

Discussion

We find that lake-stream transitions usually coincide with limnetic–benthic shifts in prey resource use. Stickleback distributed across these transitions usually exhibit divergence in foraging morphology and often form two populations substantially differentiated at neutral markers. Because this genetic differentiation is associated with contrasting foraging modes and traits, we infer progress toward ecological speciation. Coalescent-based simulations support the hypothesis of divergence with substantial gene flow, even though the temporal distribution of this gene flow remains uncertain. In addition to these generalizations, we find dramatic variation among systems in the degree of divergence between lake and stream fish. In short, replicate lake-stream transitions have yielded variable progress toward ecological speciation in a parapatric context.

SELECTION, MORPHOLOGY, AND GENE FLOW

Our first major finding is correlated divergence in foraging morphology and resource use across lake-stream transitions. The observation of shallower bodies and more numerous gill rakers in limnetic-foraging stickleback compared to benthic-foraging stickleback conforms well to the findings of previous work on stickleback (Schluter and McPhail 1992; Robinson 2000; Berner et al. 2008) and other fish (Robinson and Wilson 1994; Skulason and Smith 1995; Robinson and Schluter 2000). These phenotypic differences likely have a strong genetic basis—as inferred from previous work on lake-stream stickleback (Gross and Anderson 1984; Lavin and McPhail 1993; Hendry et al. 2002; Sharpe et al.

Table 1. Summary of IMa parameters characterizing the genetic divergence between the adjoining populations identified by STRUCTURE. Parameter estimates are averages across five replicate simulations (associated 95% confidence limit in brackets, omitted if zero) and include unidirectional migration rates (m_1=upstream, m_2=downstream) and divergence time (t) scaled by the mutation rate μ. Also given are the effective number of immigrants per generation (Nem=$m\Theta/4$), and divergence time in generations (t=t/μ) assuming $\mu=10^{-4}$. The last column gives genetic differentiation (Weir and Cockerham's 1984 F_{ST}, standardized in brackets) between the clusters. *All P=0.0001 based on 9999 randomizations.

System	m_1	m_2	t	N_em_1	N_em_2	t	F_{ST}*
Beaver	0.00	0.692 (0.139)	0.111 (0.005)	0.00	0.103	2,650	0.23 (0.73)
Boot	0.97 (0.999)	12.49 (1.59)	0.183 (0.054)	0.15	1.41	4,357	0.16 (0.36)
Joe's	236 (133.8)	231.3 (73.1)	0.007 (0.001)	2.84	47.6	168	0.12 (0.22)
McCreight	0.00	0.464 (0.028)	0.896 (0.063)	0.00	0.85	8,964	0.10 (0.58)
Pye	0.056 (0.069)	0.586 (0.083)	0.287 (0.025)	0.032	0.299	2,874	0.14 (0.58)
Robert's	168.4 (145.4)	234.4 (111.7)	0.01 (0.003)	6.47	7.0	100	0.05 (0.13)

2008) and other stickleback systems (Hatfield 1997; Robinson 2000; Peichel et al. 2001; Schluter et al. 2004; Albert et al. 2008). Divergence almost certainly occurred independently within each system, as indicated by our own genetic data, and by previous work on lake-stream stickleback that examined other microsatellite markers (Hendry and Taylor 2004) and also mitochondrial DNA (Thompson et al. 1997). (The possibility that close relatedness within systems is simply due to gene flow on secondary contact cannot be excluded definitively, as in other studies, but is highly unlikely here.) The strong and relatively consistent association between foraging morphology and resource use, despite independent origins of the different lake-stream pairs, indicates largely parallel responses to divergent selection across limnetic–benthic ecotones (see also Berner et al. 2008). These results also parallel limnetic–benthic divergence within and between lake stickleback (Schluter and McPhail 1992).

Our second major finding is that ecological and morphological divergence is often associated with discontinuities in neutral genetic variation. A negative association between adaptive divergence and gene flow can arise if either variable influences the other (Räsänen and Hendry 2008). Indeed, lake-stream stickleback have previously been used to argue that gene flow constrains adaptive divergence (Hendry et al. 2002; Hendry and Taylor 2004; Moore et al. 2007). Here, we specifically addressed the opposite causal pathway—adaptive divergence constraining gene flow (ecological speciation). As emphasized by Räsänen and Hendry (2008), inferring this causal pathway is difficult without measures of (or surrogates for) divergent selection. By providing such estimates (diet), we were here able to provide support for the causal pathway from divergent selection to adaptive divergence to reproductive isolation (for other examples, see Ogden and Thorpe 2002; Grahame et al. 2006; Seehausen et al. 2008). Our work on lake-stream stickleback thus provides evidence for both causal pathways between adaptive divergence and gene flow, further highlighting the importance of inferential approaches that allow discriminating between alternative arrows of causality (Räsänen and Hendry 2008).

Although our study demonstrates clearly that stickleback can at least maintain genetic discontinuities across ecotones, we also hoped to make inferences about divergence that originated in parapatry, that is, in the face of gene flow. Indeed, our IMa simulations consistently indicated nonzero to very substantial gene flow during divergence, at least in the downstream direction. Migration generally concentrated in the more recent half of the divergence period, however, so that some initial phase of differentiation in isolation remains a possible alternative to entirely parapatric divergence. The IMa analysis also indicated that population splitting occurred over short time spans ranging from approximately 70 to 8400 generations (generation time in stickleback is typically 1–2 years, Bell and Foster 1994).

Even though these simulation results are crude and subject to a series of assumptions, they appear biologically plausible for several reasons. First, IMa consistently indicated downstream-biased dispersal, which agrees with a priori expectations of stickleback movement in lake-outlet stream systems. Second, estimated divergence times nicely match the upper possible age limit for these stickleback populations (around 12,000 years), as inferred from geological data (Mathews et al. 1970; Clague and James 2002). Third, Caldera and Bolnick (2008) found that the postglacial colonization of lake and stream habitats within the Amor de Cosmos watershed occurred over an extended period spanning several thousand years. Substantial differences in splitting times among lake-stream systems are thus possible.

Taken together, our study supports the hypothesis of ecological speciation in the face of gene flow, although the precise spatial context of divergence remains uncertain. Impermeable dispersal barriers between lakes and streams are rare or absent today—we have inspected the entire length of most of these streams. This argues for limited opportunity for allopatric differentiation. Moreover, parapatric speciation within just a few hundred or thousand

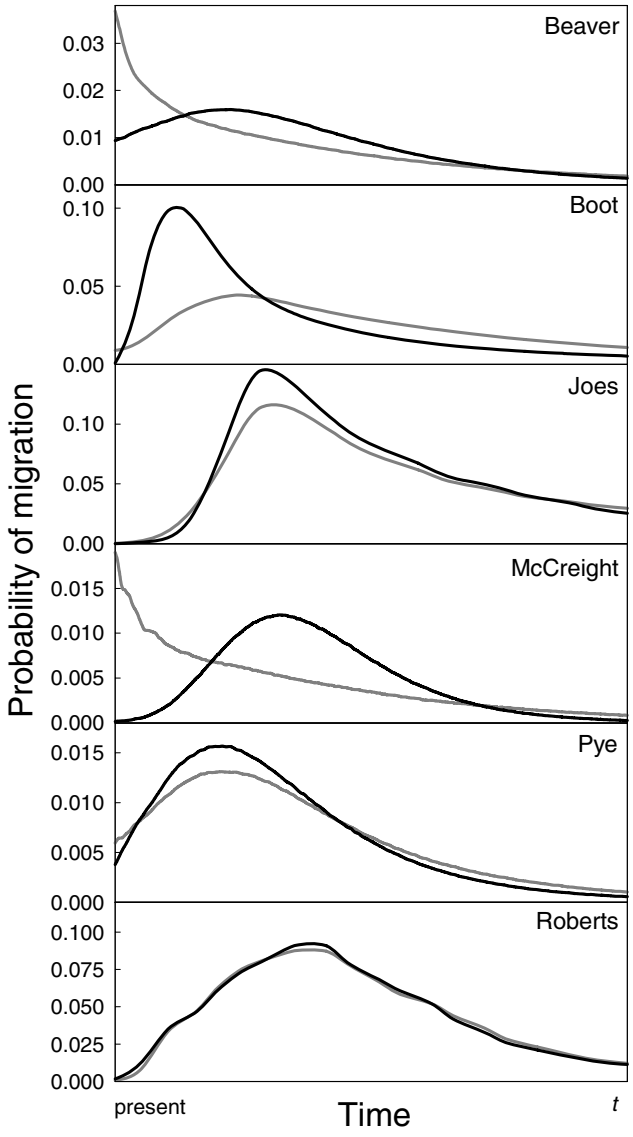

Figure 6. Probability density distributions for migration events from estimated splitting time (*t*; differs among systems, see Table 1) to the present. Gray and black curves indicate up- and downstream migration, averaged across the six microsatellite loci (weighted by the number of migration events per locus).

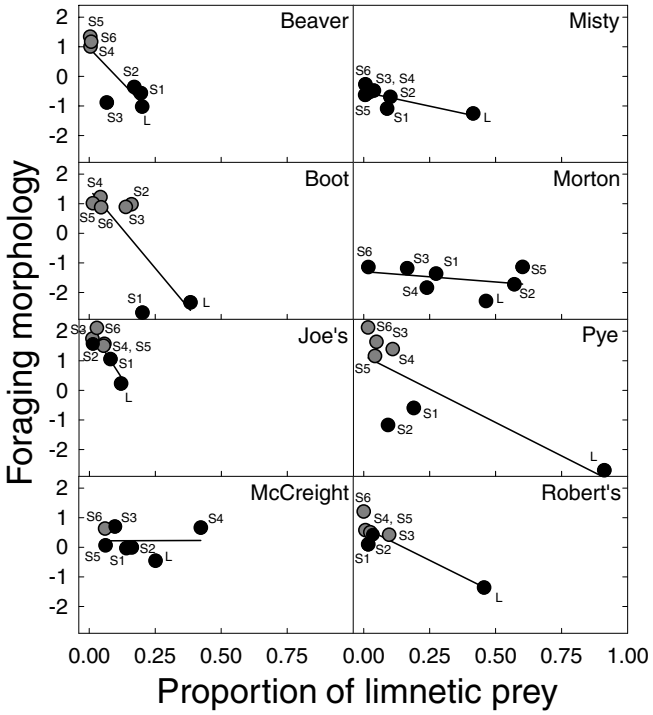

Figure 7. Stickleback foraging morphology is generally correlated with local resource use (PLP). Morphology is here expressed as first principal component extracted from the pooled sample means for body shape (RW1) and gill raker number (high PC1 scores indicate deep bodies and few gill rakers). Small letters denote the sample sites (L = lake, S1–6 = stream). Sample sites belonging to the genetic upstream and downstream clusters identified by STRUCTURE (not in Misty and Morton) are shown in black and gray, respectively. Note that the genetic clusters are usually differentiated in morphology and resource use.

generations is consistent with recent theory (Gavrilets et al. 2000; Gavrilets and Vose 2005; Thibert-Plante and Hendry 2008). On the other hand, even brief periods of initial or recurrent spatial isolation may have greatly facilitated divergence. More definitive conclusions must await the reconstruction of divergence history with higher resolution, including through the use of more extensive sequence data.

Our finding of substantial genetic differentiation between lake and stream stickleback within independent systems points to the importance of considering possible reproductive barriers. First, selection against migrants (Hendry 2004; Nosil et al. 2005) may be important, given suggestive evidence from reciprocal transplant experiments with lake-stream stickleback (Hendry et al. 2002). Second, divergent selection across the ecotone may reduce the fitness of hybrids because they are morphological intermediate (Schluter 1995; Hatfield and Schluter 1999; Hendry et al. 2002; Rundle 2002; D. Berner, K. Räsänen, A. P. Hendry, unpubl. data) and may therefore be maladapted to both environments (Schluter 1995; Rundle 2002; Rundle and Nosil 2005). Third, adaptive habitat choice may reduce movement between lakes and streams, as has been found in a recent stickleback mark–recapture experiment (D. Bolnick, L. Snowberg, C. Patenia, W. Stutz, T. Ingram, and O.-L. Lau, unpubl. data). Fourth, adaptive divergence in morphology and behavior may influence mate choice and therefore cause assortative mating, a result demonstrated for other stickleback systems (see Nagel and Schluter 1998; McKinnon et al. 2004). Finally, the parapatric nature of lakes and streams will impose a partial restriction on gene flow simply because of the spatial segregation of the habitats. Indeed, theoretical work shows that divergence in parapatry should be easier than divergence in sympatry (Endler 1977; Doebeli and Dieckmann 2003; Gavrilets 2004). In

Table 2. Genetic clustering into distinct parapatric populations within watersheds (based on STRUCTURE assignment) coincides with the exploitation of different prey resources. Fish classified to the lake cluster consume more limnetic prey than individuals belonging to the stream cluster, the difference being significant (or marginally so) in all watersheds.

System	Average PLP lake cluster	Average PLP stream cluster	Difference	P^*
Beaver	0.158	0.005	0.153	0.0001
Boot	0.336	0.081	0.255	0.0001
Joe's	0.071	0.037	0.034	0.0745
McCreight	0.189	0.060	0.129	0.0132
Pye	0.398	0.062	0.335	0.0001
Robert's	0.168	0.034	0.134	0.0001

*Based on 9999 randomizations.

short, reproductive barriers have the potential to be multifarious in lake-stream stickleback, and their relative importance is the focus of ongoing work.

FACTORS INFLUENCING PROGRESS TOWARD ECOLOGICAL SPECIATION

A key finding of our study is the tremendous variation among replicate systems in the magnitude of divergence between lake and stream stickleback. At the one extreme, systems like Misty (see also Moore et al. 2007) and Morton have produced weak differentiation in morphology and none in neutral markers, despite strong variation in selective conditions (Fig. 7). In other words, gene flow here seems to homogenize most of the genome except at some loci underlying phenotypic change (Wu 2001). At the other extreme, upstream and downstream stickleback within Beaver, Boot, and Pye would certainly qualify at least as distinct ecotypes, given the striking and correlated differentiation in diet, morphology, and neutral markers. Here, substantial divergence obviously spans most of the genome. Systems like Joes' and Robert's can be viewed as intermediate situations, with substantial genetic differentiation eventually occurring but showing a downstream displacement from the shifts in selection and phenotype (classical tension zones sensu Barton and Hewitt 1985; Jiggins and Mallet 2000).

The above variation among systems allows some refined hypotheses regarding the conditions influencing progress toward ecological speciation. Notably, both the McCreight and Morton systems stand out in that they display highly limnetic foraging at downstream sites (Fig. 2), suggesting that resource-based selection is here spatially fluctuating rather than strictly divergent. As these systems also show negligible morphological and neutral genetic differentiation (or only far downstream in McCreight) (Fig. 7), we hypothesize that divergence is here impeded by the lack of strong divergent selection (i.e., the lack of a clear-cut limnetic–benthic ecotone). This finding provides empirical support (see also Seehausen et al. 2008) for the theoretical prediction that the abruptness and magnitude of the ecological gradient is a key determinant of divergence with gene flow (Slatkin 1982; Doebeli and Dieckmann 2003; Gavrilets and Vose 2005; Thibert-Plante and Hendry 2008).

Divergence patterns also hint at dispersal rates as an additional factor influencing progress toward ecological speciation (Gavrilets et al. 2000; Hendry et al 2001). Within Joe's, Pye, and Robert's, stream sites close to the lake clustered genetically together with the lake sample despite their resident fish being quite different from the lake fish in morphology and especially in diet. One might infer that these systems would not have produced substantial differentiation at all if the stream habitat was too short for selection to finally overcome gene flow. Indeed, that particular outcome appears to have been carried to an extreme in the Misty system (J. S. Moore and A. P. Hendry, unpubl. data). Here, the outlet population occupies the shortest section of stream in any of our watersheds and shows minimal divergence in morphology and neutral markers despite striking divergence in diet. Factors influencing dispersal rates from lakes into streams might include flow rates and habitat properties (e.g., beaver dams and water depth), as well as the relative population size of lake and stream stickleback.

To summarize, our analysis of stickleback across multiple lake-stream transitions has identified variable progress toward ecological speciation in a parapatric context. This highlights that speciation resembles a tug of war between selection and gene flow with diverse possible outcomes. It remains an open question whether divergence within the eight systems will increase over time, or whether the observed stages represent stable or fluctuating selection–gene flow equilibria. We suggest that differences in progress toward speciation are partly determined by variation in the strength of divergent selection, and perhaps by dispersal rates between habitats. Testing the importance of these determinants more directly, characterizing reproductive barriers as well as their genetic basis, and analyzing the extent and history of gene flow during divergence with more powerful genetic data promises further progress toward understanding speciation.

ACKNOWLEDGMENT

Field work was supported by J. S. Moore, K. Hudson, and D. Bolnick, and accommodation was provided by Western Forest Products Inc. W. Salzburger, P. Nosil, M. Barluenga, D. Bolnick, M. Matschiner, M. Niemiller, and J. Feder provided valuable suggestions for analysis and/or comments on the manuscript, and E. Brander helped with gill raker counts. Genotyping was performed efficiently by Génome Québec (Geneviève Genaud, Alexandre Belisle). DB and ACG were supported financially by the Swiss National Science Foundation (grant

PBBSA-111216), the Janggen-Pöhn Foundation, the Roche Research Foundation, and the Stiefel-Zangger Foundation. A. H. is supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada. We are most grateful to all these persons and institutions.

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Associate Editor: J. Feder

Supporting Information

The following supporting information is available for this article:

Figure S1. UPGMA phylogram showing the genetic relationship among the 56 stickleback samples (L = lake, S1–S6 = stream labeled by increasing distance from the lake), based on six microsatellite markers and Nei's (1972) standard genetic distance.

Table S1. Geographic location of the lake (L) and outlet stream (S1–S6) sites sampled in the eight watersheds, ordered by increasing distance from the lake.

Table S2. Results from GLMs testing for change in body shape (first relative warp RW1, and multivariate), gill raker number, and prey type (proportion of limnetic prey PLP) in relation to geographic distance from the lake.

Table S3. Genetic differentiation among sample site pairings within the eight lake-stream systems.

Table S4. Results from global GLMs showing that stickleback body shape (size-adjusted RW1) and gill raker number are related to local resource use (expressed as proportion of limnetic prey, PLP).

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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