Parallel Evolution and Inheritance of Quantitative Traits

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ABSTRACT: Parallel phenotypic evolution, the independent evolution of the same trait in closely related lineages, is interesting because it tells us about the contribution of natural selection to phenotypic evolution. Haldane and others have proposed that parallel evolution also results from a second process, the similarly biased production of genetic variation in close relatives, an idea that has received few tests. We suggest that influence of shared genetic biases should be detectable by the disproportionate use of the same genes in independent instances of parallel phenotypic evolution. We show how progress in testing this prediction can be made through simple tests of parallel inheritance of genetic differences: similar additive, dominance, and epistasis components in analysis of line means and similar effective numbers of loci. We demonstrate parallel inheritance in two traits, lateral plate number and body shape, in two lineages of threespine stickleback that have adapted independently to freshwater streams on opposite sides of the Pacific Ocean. Notably, reduction of plate number in freshwater involves a substitution at the same major locus in both lineages. Our results represent only a first step in the study of the genetics of parallel phenotypic evolution in sticklebacks. Nevertheless, we have shown how such studies can be employed to test the genetic hypothesis of parallel evolution and how study of parallel evolution might yield insights into the roles of both selection and genetic constraint in phenotypic evolution.

Keywords: parallel evolution, genetics, genetic constraint, stickleback.

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> Related species will vary in similar directions and be subject to similar selective influences. They may therefore be expected to evolve in parallel. (Haldane 1932, p. 76)

Divergence is the prominent feature of adaptive radiation (Schluter 2000). Yet many adaptive radiations also incorporate a great deal of repetition in the form of parallel phenotypic evolution, defined as the independent evolution of the same trait in closely related lineages (Futuyma 1986). Discovery of parallel evolution has recently accelerated, aided by molecular and ecological studies that have detected similar but independent evolutionary transitions in traits across multiple populations or closely related species in association with changes of environment. Examples include the independent reduction of eyes in different populations of cave amphipods *Gammarus minus* (Jones et al. 1992); the origin of parallel clines in wing length in *Drosophila subobscura* (Huey et al. 2000); repeated divergence of life histories in Trinidad guppies *Poecilia reticulata* between low- and high-elevation streams (Reznick et al. 1996); multiple origins of dwarf and normal forms of lake whitefish *Coregonus clupeaformis* (Pigeon et al. 1997); parallel shifts in body size of skinks of the *Eumeces skiltonianus* complex (Richmond and Reeder 2002); multiple origins of similar *Anolis* lizard ecomorphs on Caribbean islands (Losos et al. 1998); and the repeated evolution of distinct physiological types in *Lathsenia* species distributed over soil ion concentration gradients (Rajakaruna et al. 2003).

The recognized significance of parallel phenotypic evolution lies in its contribution to the study of natural selection. Natural selection has almost certainly played a crucial role in phenotypic evolution whenever the same traits of different populations and related species evolve repeatedly and consistently in association with similar transitions in environment (Simpson 1953; Schluter and Nagel 1995; Rundle et al. 2001). Genetic drift can also yield parallel phenotypic evolution, but the transitions would not consistently be linked to change of environment. For this reason, parallel evolution is central to the

comparative investigation of ecological adaptation (Harvey and Pagel 1991; Schluter 2000).

Less well understood are the genetic implications of parallel phenotypic evolution. Haldane (1932) attributed parallel evolution not only to similar selection pressures but also to the production of heritable variation in similar directions in closely related species. This genetic component of his hypothesis incorporated two important ideas: that new genetic variation underlying phenotypic traits is biased in some directions, leading to a bias in the direction of evolution, and that these genetic biases are shared between related species, contributing to parallel evolution. Haldane made these points succinctly in a genetic context, but others have espoused related ideas. Vavilov (1922) realized that closely related species tend to vary in the same way phenotypically and that the direction of these common intraspecific variations mirror recurrent differences between species. Wake (1991), Shubin et al. (1995), and West Eberhard (2003) suggested that parallel evolution might be a reflection of developmental constraints shared between closely related species. The developmental hypothesis is linked to Haldane's genetic hypothesis if developmental mechanisms underlie biases in the production of genetic variation available to selection (cf. Maynard Smith et al. 1985; Raff 1996).

The role of genetic bias on evolution by natural selection has become widely appreciated (Futuyma et al. 1995; Schluter 1996; see review in Schluter 2000), but there have been few tests of the role of genetic constraints specifically in parallel evolution. The most common approach has been to compare developmental variations appearing within species with repetitive differences found between species (Alberch 1983; Wake 1991). Here we suggest another test of the genetic hypothesis on the basis of its straightforward consequence that separate instances of parallel phenotypic evolution should reflect the same biases in variation at underlying loci. We consider one prediction following from this consideration, that parallel trait evolution should involve independent substitutions at the same underlying loci more often than would be expected from the total number of genes influencing the trait. This expectation follows from models of gene interaction in which some genes more than others encounter obstacles to change. For example, metabolic pathway models (Rausher et al. 1999) and models of gene regulatory networks (Stern 2000) incorporate the idea that mutations in some genes have more widespread, negative pleiotropic effects on the phenotype than other genes and are therefore more likely to be conserved. If these effects are strong, transitions that do occur should be concentrated on the relatively small number of loci under fewest pleiotropic constraints. It follows that similar selection pressures on closely related lineages should lead disproportionately often to transitions at the same underlying loci if biases are shared. Indeed, any process that inhibits change in some genes more than others leads to a similar expectation if closely related species share these mechanisms. An alternative hypothesis is that no substantial genetic biases are present, in which case independent instances of parallel phenotypic evolution should rarely involve the same genes (assuming that a large number of genes participate in the development of the trait). While few would take the extreme position that genetic constraints are absent, distinguishing alternative expectations might yield fuller understanding of the processes underlying parallel phenotypic evolution.

The most direct way to detect repeated changes in the genes underlying independent phenotypic transitions is to hunt down and sequence the genes in each instance (e.g., Cooper et al. 2001). However, a simple and useful interim step is to examine whether parallel changes in the same traits in independent lineages exhibit parallel inheritance. By parallel inheritance we mean that independent lineages display similar patterns of inheritance when divergent populations are crossed, that is, similar additive, dominance, and epistasis components in analysis of line means (Lynch and Walsh 1997) and similar effective number of loci (Lande 1981; Lynch and Walsh 1997). In its strong version, parallel inheritance of population differences in closely related lineages reflects allele substitutions at the same loci. In its weak form, parallel inheritance results from allele substitutions that behave similarly in crosses whether at the same or different loci. Although the weak version of parallel inheritance will often be the best that can be established at this preliminary stage of study, analysis of crosses between independently derived phenotypes such as the complementation test can be used to gain evidence for the strong form of parallel inheritance (e.g., Wilkens 1971; Sucena et al. 2003).

We demonstrate this approach with a study of parallel evolution and inheritance of quantitative traits in the threespine stickleback (*Gasterosteus aculeatus* complex). The threespine stickleback species complex is phenotypically and ecologically diverse across its holarctic range and has representatives in a wide diversity of aquatic environments (Bell and Foster 1994). Parallel evolution in the group is extremely common, especially following the repeated colonization of freshwater by the marine ancestral phenotype (McPhail and Lindsey 1970; Bell 1974, 1976; Bell and Foster 1994; Walker and Bell 2000). In both Atlantic and Pacific oceans, marine threespine sticklebacks have well-developed pelvic girdles, long pelvic spines, and a full series of lateral bony plates extending from the pectoral girdle to the tail. In contrast, derived freshwater populations show an overwhelming tendency toward reduction of all elements of body armor (Bell and Foster 1994).

Dorsal and pelvic spines in freshwater populations are usually shorter than those of marine fish and in some cases are absent. Most freshwater populations also have few lateral plates. Finally, marine sticklebacks are more slender and fusiform than their freshwater counterparts (Walker and Bell 2000). The consistency of phenotypic changes following colonization of freshwater (i.e., parallel evolution) implies that selection is responsible for these changes (Bell and Foster 1994).

We analyze parallel reduction of armor plate number and parallel shape change in two pairs of Pacific marine and stream-resident populations widely separated in space: one in Japan and the other in British Columbia. Each stream population is independently derived from adjacent marine sticklebacks. First, we quantify parallel evolution between the two marine and stream-resident pairs in two aspects of phenotype: lateral plate number and body shape. Second, we conduct tests for parallel inheritance of differences in armor plate number and body shape using crosses between populations within and between the two geographic regions. These two traits were chosen because they are ecologically significant and differ considerably between marine and stream populations. They also probably represent the extremes of complexity of underlying genetic variation. Few genes are likely to account for transitions in plate number (Hagen and Gilbertson 1973; Avise 1976; Hatfield 1997; Peichel et al. 2001; Colosimo et al. 2004; Cresko et al. 2004). Transitions in body shape, a multidimensional trait, may involve many loci. However, suites of shape traits consistently evolve together across environmental gradients (e.g., Walker and Bell 2000), and an influence of major genes on the whole suite remains a plausible explanation for this pattern. We demonstrate parallel inheritance in both traits and implicate transitions at the same loci, the strong form of parallel inheritance, in one of the traits.

Material and Methods

Fish Populations

We examined plate number and body shape differences among four threespine stickleback populations: a stream and adjacent marine population in Canada, and a similar pair of Japanese populations. The stream populations remain in the streams year-round whereas the marine populations are anadromous, living in the sea but returning to freshwater streams to breed. Canadian stream and marine sticklebacks were collected using minnow traps from the Salmon River near its junction with the Fraser River in British Columbia. The Japanese stream sticklebacks were collected from the headwaters of Nakagawa Creek, a tributary of the Ibi River system, Honshu Island. The Japanese marine sticklebacks were from the Kushiro River, Hokkaido Island. Fish from the wild were collected at the onset of each population's breeding season in 1996. All fish used in our analyses were lab-reared first and second-generation progeny of these wild-caught threespine sticklebacks.

Molecular phylogenies of sticklebacks suggest that stream populations in different parts of the world are independently derived from marine ancestors (O'Reilly et al. 1993; Ortí et al. 1994; Thompson et al. 1997), implying that armor reduction and parallel transitions in body shape have evolved repeatedly in many different isolated populations. Both our Japanese populations are from the western Pacific Ocean mitochondrial DNA (mtDNA) lineage of threespine stickleback, whereas our Canadian populations are from the eastern Pacific Ocean/Atlantic mtDNA lineage (cf. Ortí et al. 1994).

Three F_1 hybrid crosses were created from these original samples: Canadian stream × Canadian marine ($CSCM_1$), Japanese stream × Japanese marine ($JSJM_1$), and Canadian stream × Japanese stream ($CSJS_1$). Crosses within each population were made at the same time (CS_1, CM_1, JS_1, and JM_1). These represented the first generation of laboratory crosses. In the following year we crossed fish from the first generation of laboratory crosses to create F_2 hybrids ($CSCM_2$, $JSJM_2$, and $CSJS_2$) and a second generation of pure crosses (CS_2, CM_2, JS_2, and JS_2). The numbers of replicate families made in the first generation for each cross were $CS_1 = 7$, $CM_1 = 10$, $JS_1 = 10$, $JM_1 = 10$, $CSJS_1 = 18$, $CSCM_1 = 19$, and $JSJM_1 = 20$. Numbers of families in the second generation were $CS_2 = 7$, $CM_2 = 13$, $JS_2 = 7$, $JM_2 = 6$, $CSJS_2 = 20$, $CSCM_2 = 23$, and $JSJM_2 = 15$. Adults were crossed as they reached optimal breeding condition as indicated by vivid nuptial coloration in males and greatly swollen abdomens in females. Each adult was used only once, and only one male was used per clutch of eggs.

Ten individuals from each laboratory generation and cross type were chosen for morphometric analyses (140 fish total). To preserve statistical independence, an attempt was made to sample randomly only one individual from each family. In a few cases, more than one fish was taken per family to maintain sample size; in this event, the additional fish was taken at random from a family already represented in the sample. Crosses that had more than one representative per family are CS_1 (three families), CS_2 (three families), JS_2 (three families), JM_2 (four families). In no case were more than two fish sampled from each family.

First- and second-generation fish were raised according to the procedure in Kassen et al. (1995). Using gentle abdominal pressure, eggs were removed from females and then placed in a petri dish with just enough distilled water

to cover them. Male fish were killed using carbon dioxide or MS222, and their testes were removed and macerated in the same petri dish. After a 15-min fertilization period, the eggs were placed in a separate 250-mL holding cup with a fine-mesh screen bottom and suspended over an airstone in a 10-L aquarium. Each aquarium was lined with washed blasting sand and coarse limestone gravel over an under-gravel filter. All tanks were maintained at 15° C and a 16 : 8 LD daily regime.

Following hatching, families of fish were raised separately in 10-L laboratory aquaria at approximately equal densities. At 6 mo, the F_1 fish were transferred to 50-L aquaria and kept over winter at 10° C and a 10 : 14 LD light regime. At age 1 yr they were brought into reproductive condition by increasing temperature to 15° C and light to 16 : 8 LD over 4 wk in order to create a second generation of hybrids. The fish were killed with a lethal dose of MS222 after ~18 mo for first-generation fish and ~6 mo for second-generation fish. All fish were preserved in 95% ethanol. Specimens used for morphometric analysis were then stained in a solution of 2% potassium hydroxide and alizarin red and returned to 95% ethanol.

Measuring Phenotypes

The number of lateral armor plates was measured on the left side of each fish. Counts included all staining plates from the pectoral girdle to the end of the tail regardless of plate size. Our analyses used the ln-transformation of the number of plates because this rendered the variances within parental populations homogeneous.

We took a digital photograph of each fish lying on its left side on a paraffin wax mount with a ruler placed along one edge as a reference for size. Focus, contrast, and brightness were adjusted by eye. We imported images to the Scion Image program (Scion 1998) and then digitized x and y coordinates of 12 landmarks to measure the shape of each fish. Landmarks used were a subset of those by Walker (1997) and included anterior tip of lower lip, anterior junction of the first dorsal spine with the dorsal midline (DML), anterior junction of second dorsal spine with DML, base of the first dorsal fin ray at the DML, insertion of the dorsal fin membrane on the DML, origin of caudal fin membrane on the DML, caudal border of hypural plate at lateral midline, origin of caudal fin membrane on the ventral midline (VML), insertion of anal fin membrane on VML, base of first anal fin ray on VML, anterior border of ectocoracoid on VML, and posterior edge of angular bone. Each landmark represented the convergence of two or more tissues and is the most informative type of landmark (Bookstein 1990).

We used partial warps analysis (tpsRelw version 1.31; Rohlf 2003) to analyze each specimen for body shape differences while controlling for geometric body size. The analysis first scales landmarks of every fish to a common centroid size and rotation and then computes an average shape of all the samples, called the consensus figure. The partial warps describe the amount of stretching, compressing, or bending of the plane of scaled coordinates that is needed to superimpose coordinates of all fish onto the consensus figure. The first two partial warps are in uniform directions (stretching, compressing, or shear, a displacement in a diagonal direction), and the remaining components describe nonlinear changes (bending or twisting). The full set of partial warps were saved and then used in a subsequent discriminant analysis.

Measurements of first- and second-generation fish belonging to the same parental (CS, CM, JS, and JM) were combined in every subsequent analysis. Correcting for generation did not alter the results, and we present only uncorrected analyses. We then carried out a linear discriminant function analysis on the partial warps to identify shape differences among the four parental populations. Finally, the resulting discriminant function scores were calculated for all hybrids as well. Hereafter, "body shape" refers to the first discriminant function.

Shape transitions along the first and second discriminant function were visualized using regression. We carried out univariate regressions of the x and y coordinates of every landmark onto a given discriminant function. Slopes of each pair of regressions were then plotted as vectors on the corresponding landmarks of the average fish shape to indicate direction and magnitude of landmark position change per unit of the discriminant function.

Testing Parallel Evolution

We define and test parallel evolution across similar environments using variance components of a linear model. We fitted a two-factor, fixed-effects ANOVA to armor plates and body shapes from the two stickleback lineages (Japan and Canada):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}. \quad (1)$$

The trait value of individual k is Y_{ijk}, modeled as the sum of a constant μ, the main effects of habitat i, and lineage j (α_i and β_j, respectively), the deviation $(\alpha\beta)_{ij}$ resulting from an interaction between habitat and lineage, and a random error term. Parallel evolution across similar environments occurs when a significant main effect of habitat is present and when the interaction between habitat and lineage is relatively small. We therefore tested parallel evolution with an F-test on the ratio of the mean square for habitat and the mean square for the interaction. The outcome of this test depends on the relative magnitudes of

the variance components for habitat (hereafter, A) and the interaction of habitat and lineage (hereafter, AB). In the extreme case where no interaction is present ($AB = 0$, $A > 0$), all lineages respond identically to environmental transitions. In less extreme cases ($AB > 0$, $A > AB$), lineages tend to shift in the same direction across environmental transitions but not by the same amount. These cases can be quantified by the difference between the two variance components expressed as a proportion of their total:

$$\gamma = \frac{A - AB}{A + AB}. \tag{2}$$

When $\gamma > 0$, lineages tend to respond similarly to the environmental transition, with $\gamma = 1$ indicating identical responses (see fig. A1 in the online edition of the *American Naturalist*). Parallel evolution is absent when $\gamma \leq 0$, with $\gamma = -1$ indicating opposite responses to environmental transitions by different lineages. We provided estimates of γ to indicate relative magnitude of the variance components in our tests. Methods to calculate the variance components and estimate γ are given in the appendix in the online edition of the *American Naturalist*.

Testing Parallel Inheritance

Analysis of Line Means. We tested inheritance patterns of mean differences between stream and marine populations in both armor plate number and body shape (represented by the first discriminant function). We estimated the net contributions of additive, dominance, and epistatic genetic effects on inheritance of means using joint scaling (Lynch and Walsh 1997). With additive effects alone, the mean phenotypes of the F_1 and F_2 generations are expected to be the average of the means of the two parental phenotypes. Dominance effects cause both generations of hybrids to resemble one parental phenotype more than the other. When additional epistatic effects are present, hybrid phenotypes differ unpredictably from the parental phenotypes, deviating from the additive plus dominance expectation.

The basic technique fits a multiple regression model to the sample means of two parental populations and their hybrids ("lines"):

$$\bar{z}_i = M_{i1}\mu + M_{i2}\alpha + M_{i3}\delta + e_i. \tag{3}$$

Here, $\bar{z}_i$ is the trait mean in the ith line, and the indicators M_{ij} represent the contributions of the constant (μ), additive (α), and dominance (δ) components of variation among line means. The e_i are random errors. Epistasis terms are incorporated into the error terms e_i.

For example, when analyzing line means of a stream × marine cross within one region (e.g., between the two Canadian populations), all $M_{i1} = 1$, indicating the constant expectation. The M_{i2} are indicators of the additive effects, being -1 and 1 for the two parental lines and 0 and 0 for hybrid lines (CS, CM, $CSCM_1$, and $CSCM_2$, respectively). These indicators represent the expectation under additivity that the means of F_1 and F_2 hybrids should equal the average of the parental means. The M_{i3} are the indicators of dominance effects. Here, the dominance indicators are -1, -1, 1, and 0 for CS, CM, $CSCM_1$, and $CSCM_2$ lines, respectively. Under dominance, the F_1's are expected to shift two units from the additive expectation and the F_2's by half as much.

To test parallel inheritance we modified the above model to incorporate two pairs of parental lines, one marine-stream pair from Canada and the second marine-stream pair from Japan. This involved adding a new term to the regression model to represent a possible difference between regions in the trait means. In this case we had eight means corresponding to the four parental lines: two F_1 lines (one from each region) and two F_2 lines. The indices for the constant, additive, and dominance components for JS, JM, $JSJM_1$, and $JSJM_2$ are identical to those described for the Canadian crosses in the above example. A new index was added to represent a constant difference of means between regions over all lines. Indices were 1 for the four Canadian lines and -1 for the four Japanese lines. Applying the modified model thus involved fitting the same additive and dominance coefficients to marine × stream crosses from both regions, which is the model of parallel inheritance. The modified error terms now included not only epistasis but also any differences between regions in the additive, dominance, or epistatic components of trait inheritance.

Beginning with the simplest regression model incorporating only the constant and the constant difference between regions terms, additive and then dominance coefficients were added sequentially until a good fit was obtained. Once a good fit was obtained, no further terms were added to the regression model. Failure to achieve a good fit even after additive and dominance components were included implies that epistasis is present or that regions differ in the additive, dominance, and/or epistatic components of trait inheritance. We did not further elaborate the model to separate these alternatives because we always achieved a good fit before this stage. Goodness of fit was evaluated using a χ^2 test, as detailed in the appendix.

An apparently good fit to an inadequate model might result if statistical power is low. As a precaution we repeated the analyses using a more relaxed significance level of 0.10 rather than the usual 0.05 to judge goodness of fit, but except where noted this made little difference to the results. Using plots of fitted effects and estimated

model coefficients, we also confirmed that excellent fits to data were achieved and that additional effects not included in the best-fit model must be small.

Analysis of Line Variances. We further tested parallel inheritance in marine × stream crosses by comparing line variances in a procedure analogous to the analysis of line means. We compared a constant-variance model with two other models. The first investigated the effects of hybridization per se on trait variance by allowing the F_1 and F_2 hybrids to have higher variance than the parental lines. If this model fit the data poorly, then we fitted an additional model in which the variance of F_2 hybrids was permitted to exceed that of the F_1 hybrids (and parental crosses), indicative of segregation variance in the F_2 generation. High segregation variance in the F_2's implies that trait differentiation between the marine and stream parents involves relatively few genes (Lande 1981).

The procedure for line variances fits a multiple regression model to the sample mean absolute deviations (MADs) in parental populations and in their hybrids (lines). The absolute deviations are calculated as the absolute value of the difference of each individual from the median of its line. The MAD is related to the variance (itself the mean of squared deviations), but regression-based tests of mean absolute difference between groups (analogous to a Levene's test) are more robust to departures from normality than *F*-tests of differences between variances (Sokal and Rohlf 1995). The model was

$$\bar{w}_i = M_{i1}\mu + M_{i2}\kappa + M_{i3}\varphi + e_i. \quad (4)$$

Here, $\bar{w}_i$ is the trait MAD in the ith line and the M_{ij} are indicators representing the contributions of the constant (μ), hybrid expansion (κ), and segregation variance (φ) components of variation among line MADs. The e_i are random errors. For example, in a cross between the marine and stream populations from Canada, all $M_{i1} = 1$, which represents the constant expectation, and the indicators of hybrid variance expansion, M_{i2}, are -1, -1, 1, and 1 for CS, CM, $CSCM_1$, and $CSCM_2$ lines, respectively. The M_{i3} indicators of segregation variance in the F_2 hybrids are -1, -1, -1, and 1 for CS, CM, $CSCM_1$, and $CSCM_2$ lines, respectively.

To test for parallel inheritance, we incorporated crosses from both marine × stream pairs from Canada and Japan, as in the analysis of line means, except that here we did not need an extra variable for difference between continents. The model thus fitted the same coefficients of hybrid expansion and segregation variance to marine × stream crosses from both regions, which is the model of parallel inheritance. Beginning with the simplest regression model incorporating only the constant term, hybrid variance expansion and segregation variance terms were added sequentially. Goodness of fit of models to data was evaluated using the same procedures as in the analysis of line means (see appendix).

Results

Parallel Evolution

The number of lateral plates showed a strong pattern of parallel evolution coincident with environment. The mean number of plates was very similar in the marine sticklebacks from both Canada and Japan and was reduced by almost identical amounts in the two derived stream populations (fig. 1*a*). The test of parallel evolution was conclusive ($F = 2{,}056.4$, df $= 1, 1$, $P = .014$), with the habitat component of variance overwhelming the interaction component ($\hat{\gamma} = 0.999 \pm 0.002$ SE).

Body shape has also evolved largely in parallel in the four populations, again in association with environment. The first discriminant axis based on the four parental populations put marine sticklebacks from both regions together at the lower extreme and the two stream populations at the upper extreme (fig. 2). The *F*-test confirmed parallel evolution ($F = 582.2$, df $= 1, 1$, $P = .028$), with the habitat component of variance again dominating the interaction component ($\hat{\gamma} = 0.996 \pm 0.008$ SE, $P < .0001$). This first axis accounted for 68% of the variance among population means and represented change in position of several landmarks (fig. 3*a*). An increase in body shape (linked with the transition from marine to stream environment) was associated with an increase in body depth, compression of the distance between the first and third dorsal spines, reduction in the length of dorsal and anal fins, and a smaller ectocoracoid (landmark 11).

The discriminant analysis furthermore indicated that stream populations were not identical and indeed had diverged in some respects relative to the marine phenotype, as shown by a significant difference along the second discriminant axis (fig. 2). This second axis accounted for 26% of the total variation among the population means and was associated with differences in the lengths and positions of dorsal and anal fins and with the presence of a slight upward rotation of the jaw in the Japanese stream population relative to the Canadian stream population (fig. 3*b*). The two marine populations differed slightly along the second axis, with the Canadian population resembling the Japanese stream phenotype. Overall, the pattern of evolution along this second axis was divergent rather than parallel, and the corresponding interaction component of variance was far larger than the habitat component ($\hat{\gamma} = -0.999 \pm 0.0015$ SE, $P > .999$).

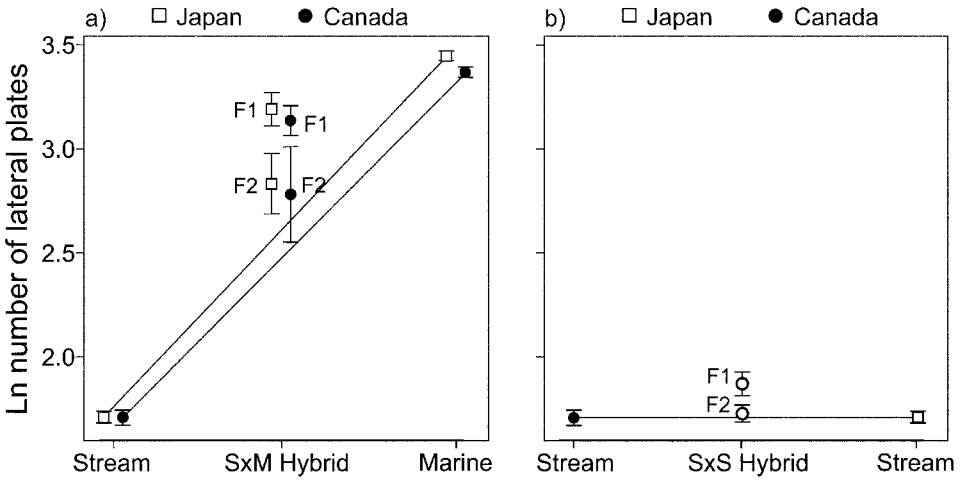

Figure 1: Means and standard errors for ln-transformed number of lateral plates in stream and marine sticklebacks and their hybrids. *a*, Crosses between marine and stream sticklebacks within regions. Lines connect parental populations. *b*, Cross between stream sticklebacks from the two regions. Line connects parental populations.

Parallel Inheritance

Lateral plate number closely followed the expectation of parallel inheritance. Differences between marine and stream populations in Japan and Canada showed similar patterns of inheritance—similar additive, dominance, and epistasis components in analysis of line means and similar components of line variances. The F_1 hybrids from both regions resembled the heavily plated marine phenotype more than the stream phenotype, and the F_2 hybrids fell between the F_1 means and the average of the two parental types (fig. 1*a*). This pattern is indicative of partial dominance, which is similar in the two regions. The additive-plus-dominance model fit the data best (table 1) with no indication of either epistasis or heterogeneity between regions.

Variances of lateral plate number in hybrid lines exceeded those of the parental species (fig. 4). The F_1 hybrids were only slightly more variable than the marine and stream parental lines in both regions, but F_2 hybrids had a mean absolute deviation roughly five times that of the parental lines. This probably represents segregation variance, implying that lateral plate number differences between marine and stream populations are determined by few genes. A model with hybrid increase and segregation variance components fit the data best (table 2), with little indication of regional differences in the pattern of hybrid variance increase (fig. 4). The overall pattern indicates that a similar number of genes determines differentiation between marine and stream environments in both Canadian and Japanese lineages.

In contrast to lateral plates, inheritance of differences in body shape (first discriminant axis) between stream and marine populations fit an additive model reasonably well (table 1). Mean body shapes of F_1 and F_2 hybrids were not significantly different from the average shapes of the parental marine and stream populations (fig. 5), and there was little indication of dominance or epistasis. There was no evidence of heterogeneity between regions, suggesting that mean body shape fits the hypothesis of parallel inheritance.

Also in contrast to lateral plates, the marine and stream difference in body shape is almost certainly polygenic. Variances of body shape in hybrid lines were significantly

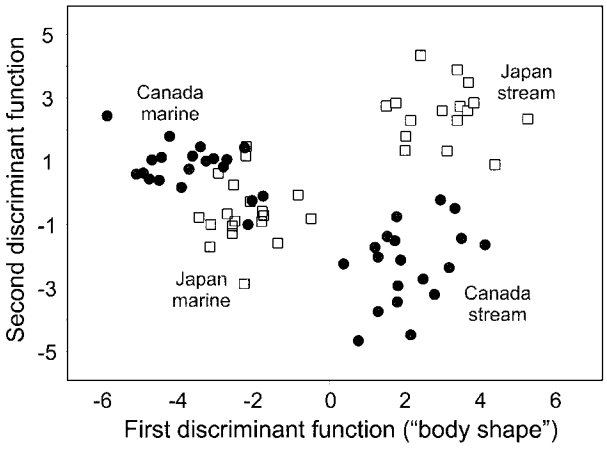

Figure 2: Linear discriminant function scores for the four parental populations based on the partial warps.

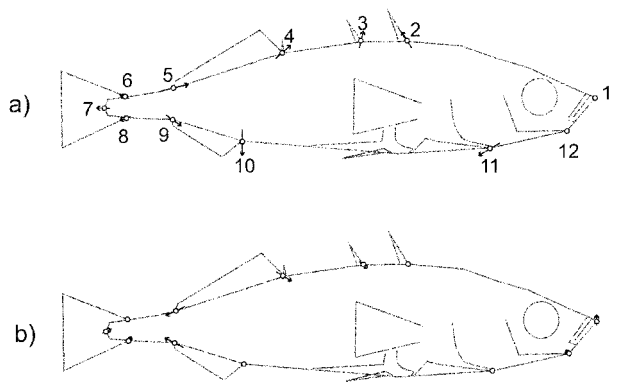

Figure 3: Shape changes along the first (*a*) and second (*b*) discriminant functions. Arrows indicate the magnitude and direction of change of each landmark per four standard deviations of the given discriminant function. Arrow direction is from marine to freshwater along the first discriminant function and from Canada stream to Japan stream along the second function (fig. 2). Landmarks are numbered as follows: (*1*) anterior tip of lower lip, (*2*) anterior junction of the first dorsal spine with the dorsal midline (DML), (*3*) anterior junction of second dorsal spine with DML, (*4*) base of the first dorsal fin ray at the DML, (*5*) insertion of the dorsal fin membrane on the DML, (*6*) origin of caudal fin membrane on the DML, (*7*) caudal border of hypural plate at lateral midline, (*8*) origin of caudal fin membrane on the ventral midline (VML), (*9*) insertion of anal fin membrane on VML, (*10*) base of first anal fin ray on VML, (*11*) anterior border of ectocoracoid on VML, and (*12*) posterior edge of angular.

greater than those of the parental species, but there was no evidence of greater variance in the F_2 generation than in the F_1 generation (table 2). Absence of segregation variance in F_2 hybrids implies that many genes probably determine body shape differences between marine and stream phenotypes. The general hybrid increase model fit the data adequately, and there was also little indication of regional differences in the pattern of hybrid variance increase (table 2). Many genes appear to determine shape differentiation between marine and stream environments in both Canadian and Japanese lineages. In this context, it is important to emphasize that "additive" inheritance of shape differences between phenotypes refers to the net effects of all genes distinguishing them, not to behavior of individual genes (Falconer and Mackay 1997).

Weak versus Strong Parallel Inheritance

In the strong form of parallel inheritance, parallel divergence events in independent lineages are determined by allele substitutions at the same loci. In the weak form, we can be certain only that parallel evolution results from allele substitutions that behave similarly in independent lineages but not necessarily at the same loci. At this point we can go no further to distinguish weak versus strong models for body shape. However, the lateral plate number trait presents two features that allow us to test these alternatives using a complementation test: dominance and simple inheritance. To this end, we carried an analysis of line means and line variances on crosses between the two stream populations from Canada and Japan.

Since reduced number of lateral plates is largely recessive in both lineages (fig. 1*a*), we expect the F_1 hybrid between stream populations to revert to the marine phenotype if different genes are involved in differentiation in the two lineages (e.g., Wilkens 1971). Since number of lateral plates is determined by few genes, we furthermore expect to see segregation variance in the F_2 generation if different genes are involved in lateral plate reduction in freshwater in the two lineages. Neither of these expectations was met. The F_1 hybrids had about as many lateral plates as did the parental stream fish (fig. 1*b*). The model of equal means could not be rejected at the standard significance level of 0.05 ($P = .08$; table 3). Even if we adopt a more relaxed significance level of 0.10 (leading to rejection of both the equal means and the additive models; table 3), it is clear that the F_1 hybrid increase is slight next to the difference between stream and marine populations (fig. 1*b*). Also, there was no indication of segregation variance in the F_2 generation: the model of equal mean absolute deviations could not be rejected in the analysis of line variances ($\chi^2 = 0.48$, df $= 3$, $P = .92$). These findings suggest that allele substitutions at the same loci are responsible for plate reduction in Canadian and Japanese stream populations.

Discussion

The idea goes back at least to Haldane (1932) that parallel evolution results in part from similarly biased production of genetic variation in close relatives. While the role of genetic bias in evolution is becoming increasingly well understood, its role in parallel phenotypic evolution has received few tests. We evaluated the prediction that influence of shared genetic biases that underlie traits should be detectable by the repeated use of the same genes in independent instances of parallel phenotypic evolution under similar environmental selection pressures. We presented quantitative methods for testing parallel evolution and parallel inheritance. We demonstrated parallel evolution and inheritance in two traits in two lineages of threespine stickleback that have adapted independently to freshwater streams on opposite sides of the Pacific Ocean. Our results represent only the first step in a study of the genetics of parallel phenotypic evolution in sticklebacks. Nevertheless, we have shown how such studies may be useful in testing the genetic hypothesis of parallel evolution, which has received less attention than natural selection.

Table 1: Line means analysis of marine × stream crosses from the two regions

	Model		
	Equal means within regions	Additive	Additive + dominance
Degrees of freedom	6	5	4
Ln(number of lateral plates)	3,837.8***	125.6***	1.9
Body shape	574.7***	7.5	…

Note: The χ^2 statistics measure goodness of fit of line means to alternative genetic models fitted sequentially. A large χ^2 indicates a poor fit. Underline indicates adequate fit of model to data.

*** $P < .0001$.

Both phenotypic traits—number of bony lateral plates and the major axis of population differentiation in body shape—exhibited parallel inheritance in our two lineages of stream and marine populations. Largely recessive alleles at the same few loci underlie reductions in the number of lateral plates in freshwater in populations from both geographical regions. In contrast, a larger number of genes of relatively small effect are implicated in the major axis of shape change, with additive net effects. The complexity of shape differences and the absence of major genes separating marine from stream populations makes further progress on the genetics of parallel evolution of body shape difficult with the methods applied herein. Possibly a trait-by-trait analysis of the components of body shape would be more fruitful than the multidimensional approach attempted here. Not all details of body shape evolved in parallel in freshwater: freshwater populations were divergent in the minor axis of population differentiation.

Our findings of few loci underlying lateral plate number differences are consistent with previous studies of threespine sticklebacks that observed high segregation variance in crosses between high- and low-plated forms (Hagen and Gilbertson 1973; Avise 1976) and with biometric and quantitative trait locus (QTL) studies that detected major loci for population and species differences in lateral plates (Hatfield 1997; Peichel et al. 2001; Colosimo et al. 2004; Cresko et al. 2004). The Colosimo et al. (2004) study found a single major QTL and three minor QTL for lateral plate number differences in a cross between a female from our Japanese marine population and a male benthic stickleback lacking lateral plates. That study also found evidence for the same major QTL in a freshwater population polymorphic for lateral plates. Reduced number of plates in several lake populations in Alaska mapped to the same linkage group as the major locus in the Colosimo et al. study (Cresko et al. 2004). The present study implicates the same genes in independent instances of lateral plate reduction in freshwater stream populations widely separated in space.

Detection of segregation variance and allele substitutions at apparently identical loci allows us to pool the data from the two geographic regions to obtain a coarse estimate of the minimum effective number of loci (Lande 1981) underlying plate reduction in freshwater. Following the approach employed by Hatfield (1997; see appendix for methods), we obtained a point estimate of 1.2 genes and a 95% confidence interval of (1.0–2.4) genes. This result is consistent with the involvement of a major locus.

The strength of support for the genetic hypothesis of parallel evolution, based on our results for lateral plate number, now depends on two conditions, neither of which can be firmly established without further data. First, the test requires that transitions at the same locus in different lineages are independent rather than merely ancestral. This criterion is assured if the alleles at the same locus have a different origin in each derived population, which can only be confirmed by gene sequencing. The situation is more

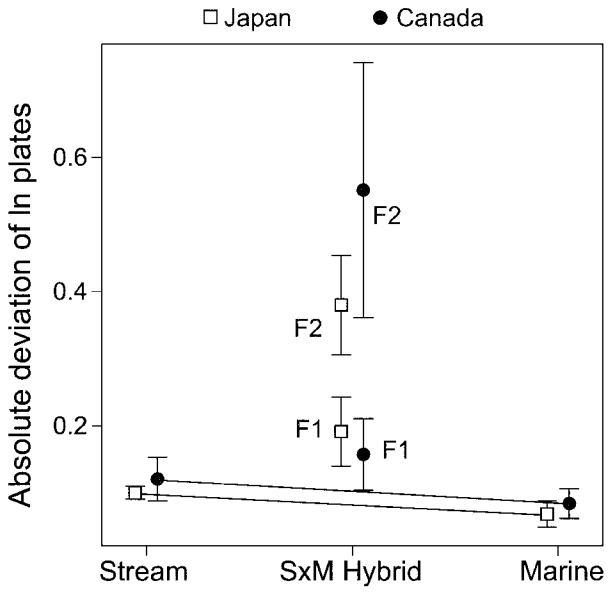

Figure 4: Mean absolute deviations of ln-transformed number of lateral plates in stream and marine sticklebacks and their hybrids (±1 SE). Lines connect parental populations.

Table 2: Line variance analysis of marine × stream crosses from the two regions

	Model		
	Equal variances within regions	Hybrid increase	Segregation variance
Degrees of freedom	7	6	5
Ln(number of lateral plates)	27.7***	12.5*	4.0
Body shape	13.4(*)	9.1	…

Note: The χ^2 statistics measure goodness of fit of line mean absolute deviations to alternative genetic models fitted sequentially. A large χ^2 indicates a poor fit. Underline indicates adequate fit of model to data.

(*) $P < .10$.

* $P < .05$.

*** $P < .0001$.

ambiguous when the same allele has gone to fixation in each derived population. It is conceivable but perhaps unlikely that the same nucleotide substitutions have arisen independently more than once (e.g., Wichman et al. 1999). More likely is that the allele was already present in the ancestral population or that it arose by mutation in one population and spread via gene flow to others (e.g., Reid et al. 2000; Daborn et al. 2002). In either case a lack of replication would prevent a test of the hypothesis of genetic bias. However, fixation of the same allele in widely geographically separated populations still begs the interesting questions of how the allele was maintained in the ancestral population across such scales and why standing variation at other loci in the common ancestor and new mutations arising since divergence have not contributed.

The second condition is that the given gene is just one of many involved in the development of the trait, such that mutations in any of these genes would also cause a change in phenotype. This assumption seems probable for a relatively late-developing trait such as the lateral plate, an organ that develops from well-differentiated dermal mesenchyme overlaid by an organized substratum of epithelium with which it may interact (Sire and Huysseune 2003). The larger the number of genes involved, the more surprising is the observation that parallel phenotypic evolution has involved the same underlying gene independently more than once.

Assuming that the above conditions are met, is genetic bias the only explanation for repeated use of the same loci underlying independent instances of parallel phenotypic evolution? As with most empirical studies of genetic constraints on adaptive phenotypic evolution, it is difficult to rule out alternative adaptive explanations for parallel inheritance. Perhaps repeated involvement of the same few loci results because mutations at these loci yield a greater phenotypic response than mutations at other loci such that they invariably experience stronger positive selection in the new environment. Or perhaps transitions at the given loci may yield greater benefits via their pleiotropic effects than transitions at other loci, again leading to disproportionately strong positive selection. Distinguishing these explanations from a hypothesis of genetic bias will require more information on the phenotypic consequences of changes at alternative loci and the mechanisms of selection acting on those changes. Nevertheless, it is difficult to avoid the conclusion that repeated use of the same loci indicates a limited number of genetic degrees of freedom underlying phenotypic evolution even if the mechanisms are still obscure (Hodin 2000).

A growing number of studies is investigating the genetic basis of parallel phenotypic evolution in nature. Shapiro et al. (2004) found that reduction of the pelvic girdle in

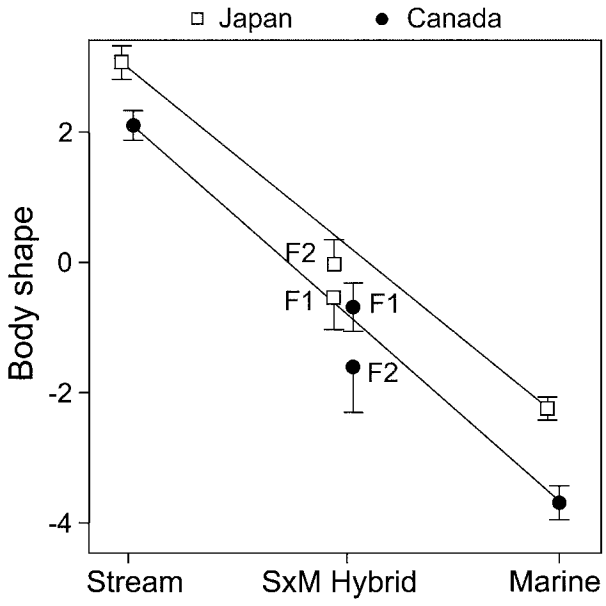

Figure 5: Means and standard errors for body shape (first discriminant function) in stream and marine sticklebacks and their hybrids. Crosses were between marine and stream sticklebacks within regions. Lines connect parental populations.

Table 3: Line means analysis of crosses between stream populations from the two regions

	Model		
	Equal means	Additive	Additive + dominance
Degrees of freedom	3	2	1
Ln(number of lateral plates)	6.8$^{(*)}$	6.8*	1.5
Body shape	12.8**	4.2	…

Note: The χ^2 statistics measuring goodness of fit of line means to alternative genetic models fitted sequentially. A large χ^2 indicates a poor fit. Underline indicates adequate fit of model to data.

$^{(*)}$ $P < .10$.

* $P < .05$.

** $P < .01$.

two lake populations of threespine stickleback, one in Iceland and one in British Columbia, is caused by changes to the same gene, *Pitx1*. Sucena et al. (2003) found that independent loss of larval trichomes in different *Drosophila* species map to the same major locus (*shavenbaby*). Gompel and Carroll (2003) found that abdominal pigment pattern in multiple *Drosophila* species usually correlated with expression of the *bab2* locus. In neither case is the adaptive significance of parallel phenotypic transitions known. In contrast, Nachman et al. (2003) found that different loci were responsible for adaptive melanism in different populations of rock pocket mice, *Chaetodipus intermedius*. Wittkopp et al. (2003) similarly found that the genes underlying differentiation of pigmentation between the sister species *Drosophila americana* and *Drosophila novamexicana* were not the same as those responsible for similar pigmentation differences in other *Drosophila* species (not all of them closely related to the pair of interest). While not a genetic study, Huey et al. (2000) found that superficially similar parallel changes in wing length in *Drosophila subobscura* involved different wing elements each time, suggesting that different genes were responsible. A classic example of multiple mutations involves repeated eye loss in *Astynax* cave fish, a partly recessive trait. Crosses between independently derived cave populations partially restored the eyed phenotype, indicating that at least some of the genes underlying eye loss are different in different populations (Wilkens 1971; Wilkens and Strecker 2003).

Most other genetic studies of parallel phenotypic evolution involve selection in natural populations in response to human alterations of environment (ffrench-Constant 1994; Schat et al. 1996; Reid et al. 2000; Daborn et al. 2002) or studies of experimental evolution in novel laboratory environments (Cooper et al. 2001, 2003). In contrast to the diversity of outcomes seen in cases of parallel evolution in nature, most examples of parallel evolution in human-modified environments seem to involve the same genes. Identical major gene loci appear to be involved in heavy metal tolerance of *Silene vulgaris* in Ireland and Germany (Schat et al. 1996). A number of cases of parallel evolution of insecticide resistance involve the same resistance allele that may have spread among populations by gene flow (e.g., Raymond et al. 1991; ffrench-Constant 1994; Daborn et al. 2002). Several mobile virulence factors appear to have transferred between separate strains of *E. coli* (Reid et al. 2000). Loss of ability to catabolize D-ribose in replicate experimental populations of *E. coli* involved the independent loss of function of the *Rbs* gene in all of 12 lines established from a common starting clone (Cooper et al. 2001).

However, different genes are also found in genetic studies of parallel evolution in natural populations responding to human alterations of environment and of parallel evolution in laboratory environments. The case of parallel evolution of gene expression levels in experimental populations of *E. coli* is especially illuminating (Cooper et al. 2003). Expression of a large suite of genes was found to differ in virtually identical ways from the common ancestral clone in both of two lines of *E. coli* propagated for 20,000 generations in a novel environment. In one line the majority of changes were wrought by a single mutation in the *spoT* regulatory gene. Remarkably, a mutation in a different regulatory gene caused the same expression changes in the second line. Because the independent lineages began from a single clone, parallel evolution involved the fixation of new genetic variation rather than standing variation. Investigation of 10 other clones revealed that eight of 12 lineages experienced independent fixation of new mutations in *spoT*, although parallel evolution of expression profiles has not been confirmed in the additional 10 clones.

Finally, there are known instances of experimental populations undergoing genetic changes at the same loci, but parallel phenotypic evolution itself was not measured (Bull et al. 1997; Crill et al. 2000). However, Wichman et al. (1999) found partial parallel evolution at the genetic level

in two experimental lines of phage adapting to a novel host environment.

In summary, the importance of parallel phenotypic evolution to studies of natural selection is well understood, but its importance to studies of genetic biases in the evolutionary process has not been widely investigated. Distinguishing genetic bias from selection would provide useful insights into the role of genetic factors on parallel evolution. Indeed, parallel phenotypic evolution might be a natural experiment for addressing the wider role of genetic constraint and selection in adaptive evolution.

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