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# Historical and ecological correlates of body shape in the brook stickleback, *Culaea inconstans*

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Using geometric morphometric methods, we evaluated the correlation between phenotypic variation and available historical and habitat information for two genetically differentiated, allopatric lineages of a widespread North American species, the brook stickleback (*Culaea inconstans*). The results obtained revealed strong patterns of structured phenotypic differentiation across the species range with extreme phenotypes occurring at the northwest and southeast range boundaries. Shape variation was broadly congruent with the distribution of two mitochondrial DNA lineages; a deep-bodied eastern form (Atlantic refugium) and a slim-bodied western form (Mississippian refugium); however, the two forms were not lineage-specific and phenotypic cladistic diversification is likely to be an artefact of underlying clinal variation associated with longitudinal and latitudinal gradients. In addition, we found little evidence of diagnosable lake and river forms across North America. Taken together, large-scale patterns of phenotypic diversity observed in *C. inconstans* suggest that relatively recent factors, such as continually varying natural selection across the range and/or potential local gene flow, may substantially mitigate the effects of historical separation or a generalized adaptive response to alternative habitats. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **96**, 769–783.

**ADDITIONAL KEYWORDS:** adaptation – allopatric divergence – cline – *Gasterosteus aculeatus* – gene flow – geometric morphometrics – morphological evolution.

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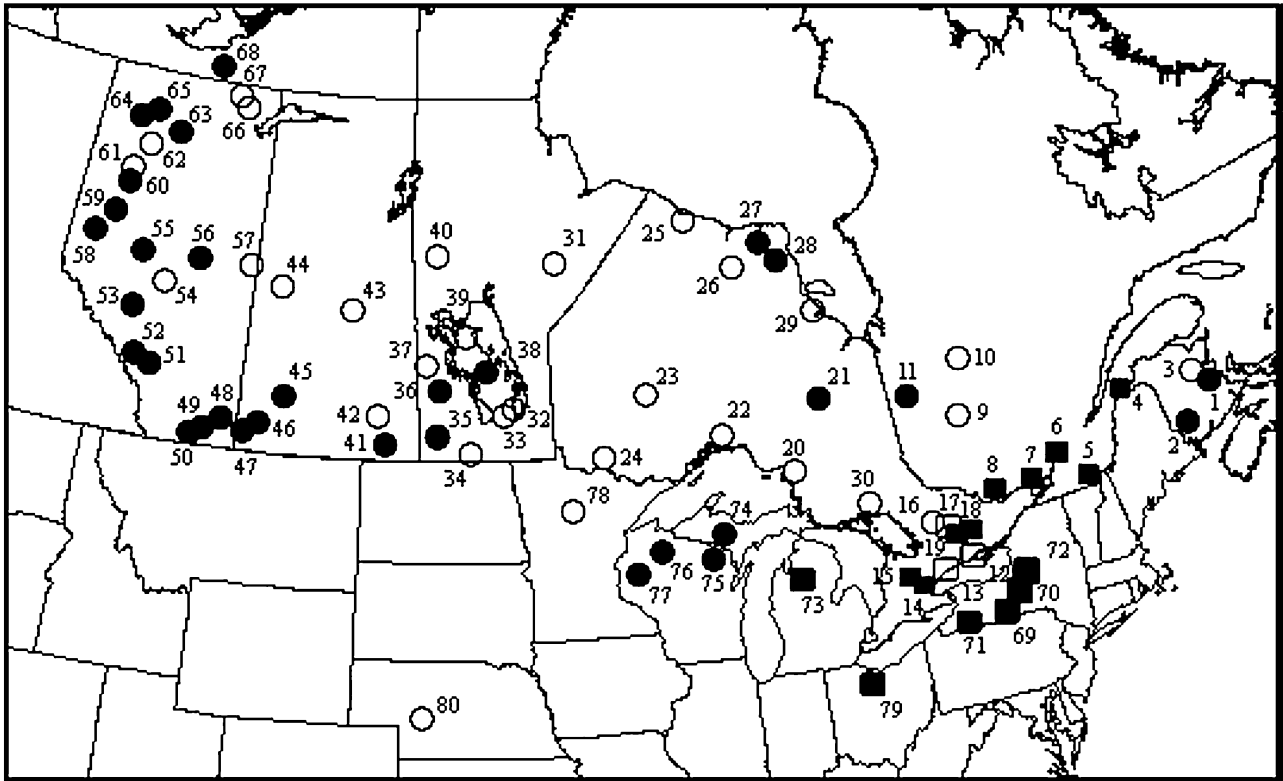
## INTRODUCTION

In most species, morphology has likely been shaped by a combination of random genetic mechanisms, such as drift and mutation (Wright, 1931; Clegg *et al.*, 2002), and selective forces, such as directional selection due to ecological factors (Orr & Smith, 1998) or selection for phenotypic plasticity (Robinson & Parsons, 2002). Integrating molecular approaches with analyses of phenotypic variation can provide insights into the relative contributions of selection and drift to population differentiation (Steinger *et al.*, 2002; Chaves *et al.*, 2007; Keeley, Parkinson & Taylor, 2007) and untangle the effects of multiple evolutionary time scales (Thompson, Taylor & McPhail, 1997). In straight-forward cases, patterns of morphological divergence are consistent with population genetic

structure and marked environmental features, which suggest the accumulation of differences as a by-product of allopatric isolation (Domingues *et al.*, 2007; Pauly, Piskurek & Shaffer, 2007; Vredenburg *et al.*, 2007) and/or adaptive divergence correlated with an identifiable habitat or niche shift (Pfenninger, Nowak & Magnin, 2007). One of the best-known examples of the latter is the freshwater benthic/limnetic and lake/stream species pairs of the threespine stickleback, *Gasterosteus aculeatus*, on the west coast of Canada (Thompson *et al.*, 1997; Taylor & McPhail, 1999; Hendry, Taylor & McPhail, 2002; Spoljaric & Reimchen, 2007). However, genetic markers and morphological traits often differ somewhat in their reconstruction of evolutionary histories. The nature of the discordance between phenotypic and genotypic patterns can be used to determine the relative importance of different, or multiple, evolutionary mechanisms (Clegg *et al.*, 2002; Pizzo *et al.*, 2006).

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**Figure 1.** Collection sites of *Culaea inconstans* used in the present study, with reference to each locality's lineage affiliation and major habitat type. Squares, Atlantic lineage; circles, Mississippi lineage; black symbols, Creek/River sites; open symbols, lake sites. Numbers correspond to the locality designations used in the Supporting information (Appendix S1).

For example, a high degree of genetic divergence in the absence of concordant phenotypic differentiation may be indicative of stabilizing selection between genetic lineages inhabiting similar niches (Santos *et al.*, 2006; Stockman & Bond, 2007) or homogenizing gene flow (Moore *et al.*, 2007).

The brook stickleback, *Culaea inconstans*, is an ideal species for investigating the interaction between genetic and morphological diversification. Currently, *Culaea* is considered to be a single, widespread, highly morphologically variable species, but mitochondrial (mt)DNA phylogeographic analyses have identified two distinct allopatric lineages (9.2% sequence divergence) that have been separated for 3.6–4.8 Myr (Gach, 1996; Mattern, 2006), which correspond to a south-eastern group and a predominantly western group (Fig. 1). Range boundaries inferred from haplotype distributions suggest that the smaller southeastern group is restricted to the Lower Great Lakes and drainages of the St Lawrence River (Atlantic clade), whereas the western group is largely composed of populations inhabiting the Upper Great Lakes, McKenzie, Mississippi, Nelson, and Hudson Bay drainages (Mississippi clade). The current widespread distribu-

tion of brook sticklebacks in North America appears to represent discrete evolutionary histories of Mississippi and Atlantic Pleistocene refugial use and subsequent northward post-glacial colonization.

A moderate amount of behavioural variability has been documented between individual populations (Burks *et al.*, 1985; Ward & McLennan, 2006; McLennan & Ward, 2008), as well as morphological variability in the number of dorsal spines (Nelson, 1969; Moodie, 1977; Edge & Coad, 1983), and the occurrence and development of the pelvic girdle (Nelson, 1969; Nelson & Atton, 1971; Nelson & Paetz, 1974; Reist, 1981), the latter of which has a known genetic basis (Nelson, 1977). In an elegant study of morphological variation, Nelson (1969) reported variation in body depth in 24 populations of brook stickleback, noting that populations from the eastern portions of North America were deeper bodied than those from the west. Additionally, body shape and size has been found to differ between populations within the same watershed associated with different types of predator communities (Moodie, 1977; Zimmerman, 2006, 2007), suggesting that there is an ecological component to morphological differentiation.

Recent advances in the study of biological shape variation (geometric morphometrics; Adams, Rohlf & Slice, 2004) permit higher resolution of the patterns of phenotypic differentiation in shape than were previously possible (O'Reilly & Horn, 2004). To date, however, a large-scale analysis of phenotypic variation using geometric morphometrics has not been undertaken in the brook stickleback and, thus, the present study aimed: (1) to examine the extent and pattern of morphological variation in body shape of *C. inconstans* across the range of the species (80 populations) using landmark based geometric morphometric methods and (2) to determine whether there are morphological correlates of the two genetic lineages (i.e. do changes in morphology reflect the deep genetic split in this species?)

## MATERIAL AND METHODS

### STUDY POPULATIONS

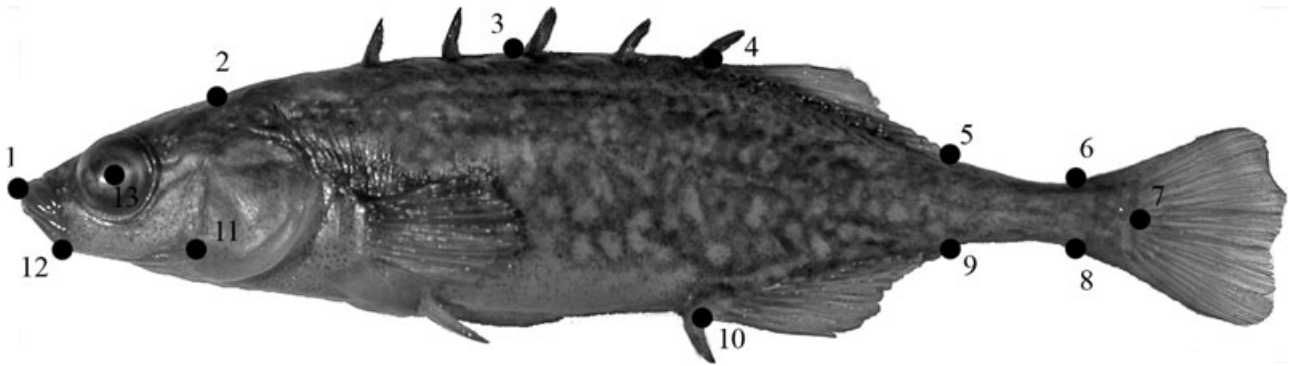
A total of 1420 whole, preserved *C. inconstans*, collected from 80 localities spanning most of the range of the species were analysed (Fig. 1). Twenty-five of the populations that were used in the present study (368 fish; see Supporting information, Appendix S1) were previously used to construct the molecular hypothesis of lineage differentiation and delimit range boundaries (Mattern, 2006). These populations were collected across the range of the species (New Brunswick to Alberta), with concentrated coverage of the Great Lakes Basin. We increased the geographic detail of the study by obtaining an additional 1052 specimens from 55 additional localities. These individuals were borrowed from the Canadian Museum of Nature (CMN); The Royal Ontario Museum (ROM); and the University of Alberta Museum of Zoology (UAMZ), University of Alberta, Canada. All individuals had been collected during the breeding season (April to September). Because *C. inconstans* generally exhibits a lifespan of 1 year (Winn, 1960; Wootton, 1976; Moodie, 1986), all of the individuals used in the present study were approximately the same age (Walker, 1997). Depending on the museum, the specimens had been preserved in 70% ethanol or in isopropyl alcohol and later transferred to 70% ethanol. Locality information, museum lending source, and gross habitat information for all population samples used in the study are provided in the Supporting information (Appendix S1). *Culaea inconstans* museum specimens were assigned to either the Mississippi or the Atlantic clade based upon locality information, resulting in a total of 286 Atlantic fish and 1134 Mississippi fish available for study. The disparity in sample sizes is reflective of the relative proportions of the total range space occupied by each group;

more Mississippi lineage populations were required to adequately represent the variability across the larger geographic range of this clade (Fig. 1). A preliminary analysis conducted with equal numbers of populations from both lineages, however, generated the same patterns of results. All analyses of shape differences were undertaken upon the 25 genetically sequenced populations (referred to as the subset analysis), as well as the complete set of 80 populations available (expanded analysis). We report the results obtained from both analyses for comparison where applicable.

Brook sticklebacks are habitat generalists that occupy shallow, heavily vegetated areas along banks and shores (Winn, 1960; Reisman & Cade, 1967; Nelson, 1969; Wootton, 1976). Because individuals used in the present study were collected from streams, creeks, and ponds, as well as along the marshy shorelines and outlets of lakes, we incorporated broad level environmental effects into our analysis by classifying each population as being either: (1) low-to-mid-order river (streams and creeks) or (2) lake, according to field and museum collection records that were verified using satellite imagery and literature accounts. By including environment type as an explanatory variable, the aim was not to correlate aspects of body shape with fine-grained environmental features (i.e. substrate, macrophyte density, dominant predator regimes, etc.) but, instead, to investigate the overall influence of these two environments on body shape.

### GEOMETRIC MORPHOMETRICS

We photographed the left lateral side of each specimen using a Nikon Coolpix 995 camera mounted directly above the fish at a height of 16 cm. Fish were photographed against a white plexiglass plate fitted with a 0.5 cm (maximum depth) graduated groove that provided room for the rounded abdomen and permitted the visible side of the fish to be parallel with respect to the plate. Dissecting pins were used to manipulate fins and spines, and straighten the fish as necessary. A standardized ruler, graduated to 1 mm, was placed above each specimen for scale. Photographs were taken under a constant light source provided by an Optix Fiber-lite series 180 high-intensity illuminator. Image distortion was minimized by centering individuals in the field of view and maintaining constant image proportions (Zelditch *et al.*, 2004). Gravid females were excluded from analysis; however, males and nongravid females were combined because the sexes cannot be distinguished based on external morphology (Reisman & Cade, 1967; Nelson, 1969) and the large number of specimens prevented us from confirming sexual identity through dissection.



**Figure 2.** Positions of thirteen anatomical landmarks digitized on photographs of *Culaea inconstans* from across the species' range ( $N = 1420$ ). 1, anterior tip of the lower lip; 2, supraoccipital notch; 3, the insertion of the medial dorsal spine at the dorsal midline; 4, anterior junction of the dorsal fin at the dorsal midline; 5, posterior junction of the dorsal fin at the dorsal midline; 6, insertion of caudal fin at the dorsal midline; 7, caudal border of hypural plate at the lateral midline; 8, insertion of caudal fin membrane on ventral midline; 9, posterior junction of anal fin membrane on ventral midline; 10, anterior junction of anal fin membrane on the ventral midline; 11, posterior angular tip of the preoperculum; 12, posterior edge of the angulo-articular; 13, center of pupil.

Thirteen anatomical landmarks were digitized on each specimen (Fig. 2) using TpsDig2, version 2.10 (Rohlf, 2004). Two additional landmarks were digitized on the ruler at a distance of 1 mm to account for scale. Landmark selection followed that previously established for the threespine stickleback, *G. aculeatus* (Walker, 1997; Spoljaric & Reimchen, 2007), modified for *C. inconstans* as needed. All of the landmarks, except for the eye (landmark 13), were distally placed on the body (Fig. 2). In the scope of the present study, both the dorsal and the pelvic spines violated the criterion of homology (Bookstein, 1991; Zelditch *et al.*, 2004). Approximately 10% of the study populations, all from the western boundary of the species, either completely lacked the pelvic girdle or were polymorphic for this trait (Nelson 1969, 1977; Nelson & Atton, 1971), so we excluded the pelvic spine as a potential landmark (Adams, Rohlf & Slice, 2004). The magnitude of shape change reported in our study might therefore be underestimated in the ventral region. The modal number of dorsal spines reported for *C. inconstans* is five; however, most populations of brook stickleback are polymorphic for dorsal spine number (from four to six; Hansen, 1939; Lawler, 1958; Nelson, 1969; Moodie, 1977; Edge & Coad, 1983). As a result, using a fixed choice of spine (e.g. the third) was deemed inappropriate. In response to this problem, we investigated spinal architecture structure using five polymorphic populations of brook stickleback from Saskatchewan. All of the specimens ( $N = 62$ ) were obtained from the University of Alberta Museum of Zoology, had previously been cleared and stained with alizarin, and were not used in the main study. In the vast majority of the stained and cleared brook sticklebacks that we

examined, the centermost spine (second, third, and fourth spines for four-spined, five-spined, and six-spined individuals, respectively) was associated with interneural space between the eighth and ninth neural spine (between 80–100% of individuals from each spine class). Where deviations from this association were observed, it was almost always only by a single neural spine and had no discernable effect on dorsal spine placement. Consequently, in the main study, these spines were considered homologous, repeatable landmarks that marked maximum dorsal depth at the same point along the spinal cord.

Landmark coordinates and partial warp scores for all specimens were calculated using CoordGen6 and CVAGen software, which are available in the Integrated Morphometrics Package (IMP) software series (H. David Sheets, Department of Physics, Canisius College, Buffalo; <http://www2.canisius.edu/~sheets/morphsoft.html>). Procrustes general least squares superimposition procedures were used to compute the mean consensus shape of all specimens, which eliminates nonshape variation by scaling, translating and rotating each figure to minimize the sum-of-squared distance of all individuals (Rohlf, 1990; Dryden & Mardia, 1998). Affine (uniform) and non-affine (partial warp) scores for each individual (Bookstein, 1991) were calculated from the superimposed landmarks and used as shape variables in subsequent analyses. Centroid size, expressed as the square root of the summed squared distances of all landmarks from their centroid (Bookstein, 1991), was calculated and used as an index of individual body size. Finally, we visualized the shape change by generating thin-plate spline deformations and magnifying the resulting vectors as necessary.

## STATISTICAL ANALYSIS

Shape variables (i.e. uniform scores, partial warp scores, and centroid sizes) were imported into SPSS, version 13, for further statistical analysis. Measurement repeatability was assessed prior to analysis by randomly selecting 18 specimens and analysing each individual's photograph twice (Arnqvist & Martensson, 1998). The congruence between the two sets of data in size (centroid size) and shape (relative warp scores) was inspected for both measures and repeatability was found to be high (Pearson's  $R > 0.93$ ).

The extent of shape differentiation between the Atlantic and Mississippi clades, and the relative contributions of genetic and environmental effects in explaining morphological variation, was tested using partial warp and uniform scores as dependent variables in a nested multivariate analysis of covariance (MANCOVA), specifying clade (Atlantic, Mississippi), environment (lake, river), and the clade  $\times$  environment interaction term as fixed effects (Claude *et al.*, 2003). In addition, the influence of unique population history was estimated by including the effect of individual population nested within the genetic lineage  $\times$  habitat interaction (Langerhans & deWitt, 2004). Centroid size was specified as a covariate because multivariate regression of partial warp scores on centroid size determined that body shape varied with size within both the Atlantic ( $R^2 = 0.24$ ,  $P < 0.001$ ) and Mississippi ( $R^2 = 0.27$ ,  $P < 0.001$ ) groups, as well as within *C. inconstans* collected from river ( $R^2 = 0.36$ ,  $P < 0.001$ ) and lake ( $R^2 = 0.22$ ,  $P < 0.001$ ) environments. The influence of each effect on morphological variation, relative to the other factors in the model, was estimated using Wilks' partial variance statistic ( $\eta^2$ ).

The assumption of homogeneity of slopes was not violated in the subset analysis of genetically sequenced populations, although it was violated in the preliminary MANCOVA model for the expanded analysis (Habitat  $\times$  Centroid size:  $F_{22,1316} = 1.69$ ,  $P = 0.02$ ; Lineage  $\times$  Centroid size:  $F_{22,1316} = 2.44$ ,  $P < 0.001$ ). Effect sizes, however, were extremely small (partial variance: 4% and 3% for lineage and habitat, respectively), and plots of centroid size against canonical scores for each effect confirmed that morphological differences between lineages and environments were not affected by centroid size. Because it is likely that the significant interaction terms are a statistical consequence of large sample size ( $N = 1420$ ) (Quinn & Keough, 2002), they were not included in the final model. A set of canonical axes for genetic, environmental, and population level effects were derived and the separation of groups in multivariate space was described as a function of the linear combination of shape variables (partial warps) that provide the greatest discriminatory ability. A subsequent dis-

criminant function analysis was performed using jackknife resampling methods to determine the accuracy with which a given individual could be identified according to genetic group (clade), environmental (habitat) group, or both.

In addition to estimating the relative influences of genetic and environmental effects on body shape, we used the IMP software program, PairDisparity6, to compare the degree of morphological diversity in body shape that exists within each clade, specifying the 80 populations as subgroups within the two clades. Within-clade disparity ( $D$ ) was estimated according to the formula:  $D = \Sigma (d_i^2)/(N - 1)$ , where  $d_i$  is the procrustes distance of the centroid of group  $I$  from the centroid of all  $N$  groups (Foote, 1993). The significance of the difference in morphological disparity was assessed via permutation test (1000 permutations).

Finally, the extent of clinal variation in shape and size was determined by Pearson correlation of canonical centroids derived from the population effect (shape) and mean population geometric centroid size (size) on latitude and longitude coordinates, respectively.

## RESULTS

Using all 1420 individual fish, the nested MANCOVA detected significant differences in brook stickleback morphology attributable to genetic lineage, habitat type and the lineage  $\times$  habitat interaction. In addition, body shape was found to vary among populations and with increasing centroid size (Table 1). Lineage affiliation accounted for the greatest percentage of partial variance (51%), followed by population nested within the lineage-by-habitat interaction (25%). In contrast, habitat type and the interaction between lineage and habitat had comparatively small effects on body shape (12% and 7%, respectively), which suggest that general morphological differences between clades might be investigated without being overwhelmed by environmental effects.

Reanalysis of the data set using the subset of twenty-five populations that had been genetically sequenced generated a similar pattern of results. Again, all effects were found to be significant and genetic differences (i.e. clade affiliation) explained a higher percentage of the variation than habitat (29% versus 11%). Centroid size again explained a high proportion of the morphological variation relative to the other factors in the model (29%). The lineage  $\times$  habitat interaction had a greater proportional influence on morphological variation than in the expanded analysis (28%), and the influence of individual population history on body shape relative to the other factors was lower (17%), which is not unexpected given the smaller sample of individual

**Table 1.** Results of expanded multivariate analysis of covariance (80 populations) of body shape between two genetic clades of brook stickleback, *Culaea inconstans*, collected from both lake and river environments

Factor	<i>F</i>	d.f.	<i>P</i>	Wilks' partial $\eta^2$ (% variance explained)
Lineage	63.34	22,1318	< 0.001	51%
Habitat	7.93	22,1318	< 0.001	12%
Lineage $\times$ Habitat	4.79	22,1318	< 0.001	7%
Centroid size	15.22	22,1318	< 0.001	20%
Population(Lineage $\times$ Habitat)	5.90	1672,28032.82	< 0.001	25%

*F*-values are based on Wilks' lambda, and the partial variance explained was estimated using Wilks' partial  $\eta^2$ .

populations. Because the two main factors of interest, lineage affiliation and habitat type, showed similar patterns of explanatory influence relative to one another in both analyses, in the remainder of the results, we graphically present the data obtained using the complete data set of *C. inconstans* available for study ( $N = 1420$ ) and include the results the subset analysis where application for comparison.

#### EFFECTS OF HABITAT ON BODY SHAPE

Large-scale habitat effects explained relatively little of the partial variance in body shape (Table 1) and discriminant function analysis misclassified *C. inconstans* from rivers and lakes approximately 30% of the time in both the expanded and subset analyses. In addition, there was little evidence for a biologically significant genotype  $\times$  environment interaction at a whole-range level of analysis (lineage affiliation  $\times$  habitat type: 7% of the variation in body shape explained). Discriminatory ability was not improved by incorporating both genetic and environmental information in either analysis (62% and 55% of fish correctly identified in expanded analysis and subset analysis, respectively) so we did not investigate habitat effects or the interaction term further (Collyer & Adams, 2007).

#### MORPHOLOGICAL DISPARITY WITHIN AND BETWEEN CLADES

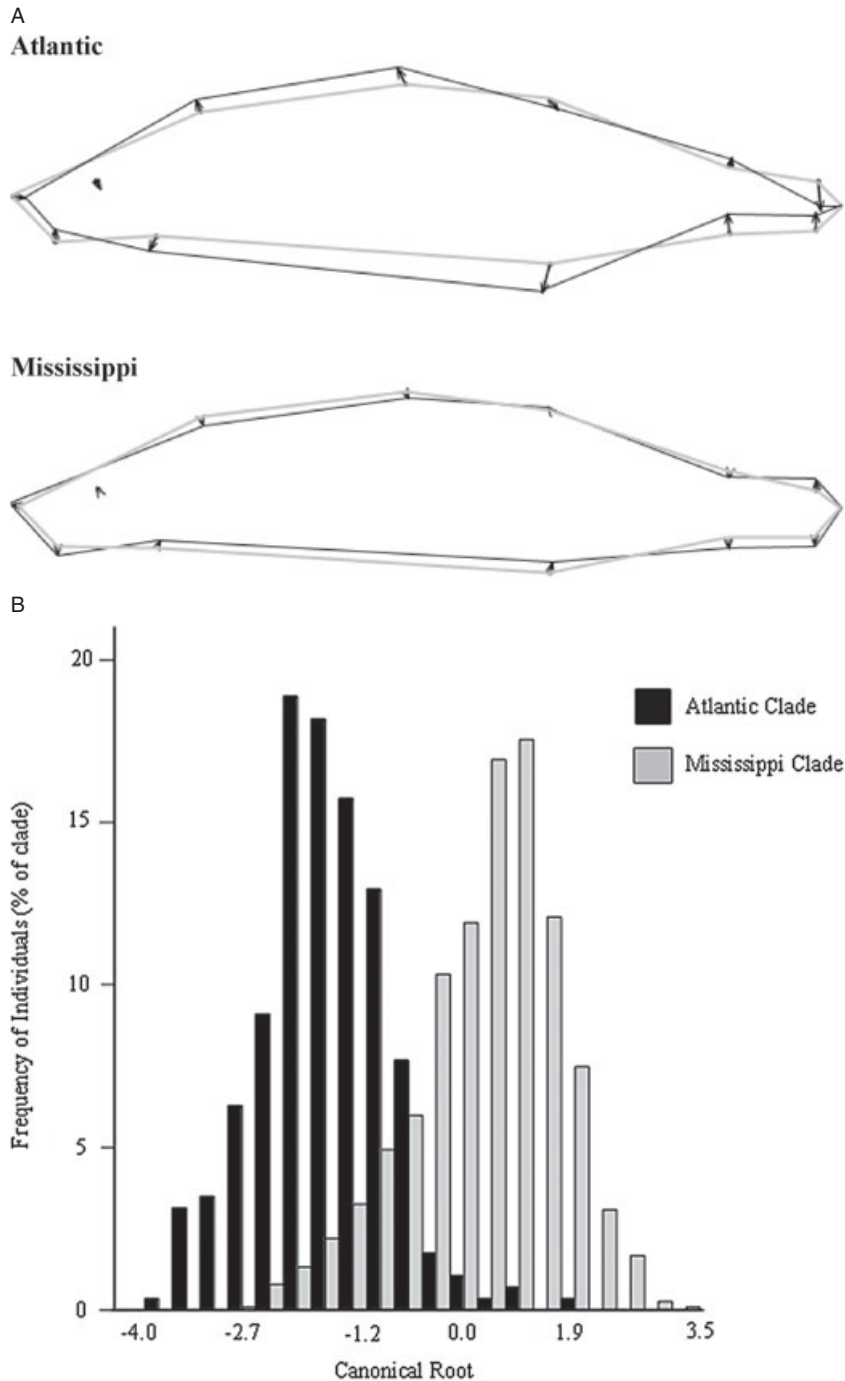
Canonical variates analysis indicated that *C. inconstans* in the Atlantic clade (i.e. lower Great Lakes and St Lawrence system) are deeper bodied, and have a more compressed head and a shallower caudal peduncle relative to their Mississippi clade counterparts. Discriminant function analysis correctly classified individuals according to lineage 72% of the time in the sequenced subset analysis. Discriminatory ability was improved by incorporating more populations (86% correctly classified in the expanded analysis). When errors occurred, the Mississippi lineage

fish were slightly more likely to be misclassified than Atlantic lineage fish (expanded analysis: 14% versus 10%; subset analysis: 29% versus 27%).

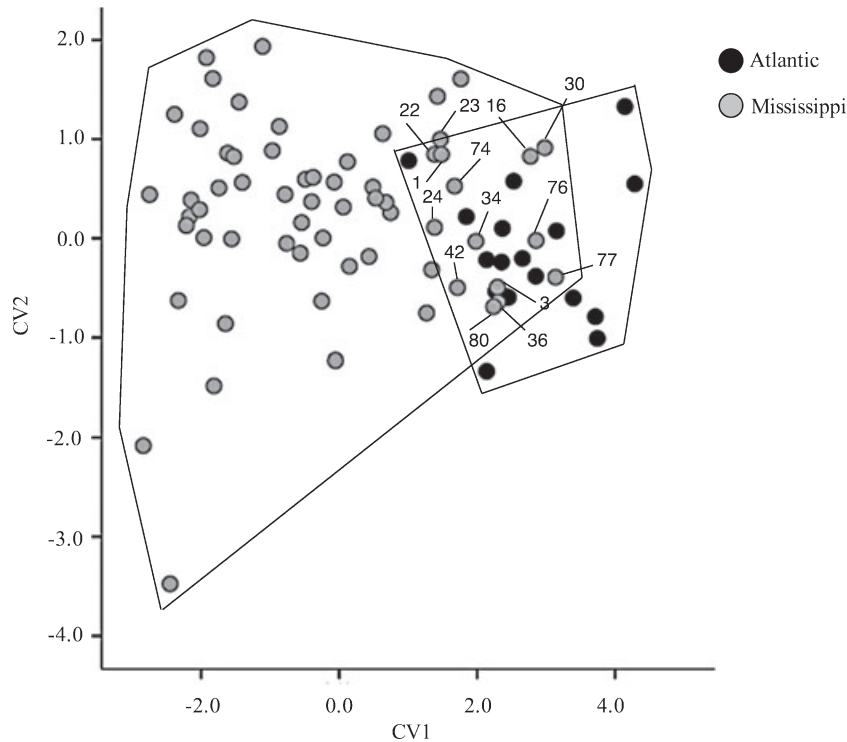
At the whole-range level of analysis (80 populations), a substantial proportion of the total variability in the morphospace corresponded to within-group variability. Subsequent investigation revealed that the degree of within-group morphological diversity was not significantly different between the clades (Foote's observed disparity: 0.0006 versus 0.0005 for Mississippi and Atlantic clades, respectively; permutation test, 1000 permutations,  $P = 0.28$ ). Thus, *C. inconstans* from the Mississippi and Atlantic regions have different mean body shapes and the range of variation in shape away from those respective means is approximately equal. Thin-plate spline visualizations of mean body form, constructed at the canonical centroids of each hypothesized clade, are presented in Figure 3A, in addition to the distribution of individuals from the two lineages in multivariate discriminant space (Fig. 3B). Although the MANCOVA indicated that the two lineages differ with respect to body shape, the two clades were not well separated along the canonical axis, demonstrating substantial overlap in body shape between the two lineages.

#### INTERPOPULATION AND CLINAL VARIATION IN SHAPE

Individual population variation was greater in the expanded analysis than the genetic subset analysis, likely due to the greater numbers of populations being tested. Across the entire range of the species, after the effect of lineage affiliation, individual population history explained the majority of the observed shape difference (25%); however, canonical variate analysis indicated that there is considerable overlap in morphospace between groups (Fig. 4). With few exceptions, the populations in the region of overlap were found to be those located along the hypothesized border between the two clades, within the Great Lakes Basin. Pearson correlations between canonical centroids for the first canonical variate (CV) derived



**Figure 3.** A, Mean body shape of Atlantic and Mississippi *Culaea inconstans*, represented by thin-plate spline visualizations constructed at the respective canonical group centroids for each clade (black lines), and contrasted with the mean common reference shape calculated from all available specimens (grey lines). Shape change is magnified by a factor of six and arrowheads indicate the direction of change, relative to the common reference shape. B, Canonical separation of *C. inconstans* from two genetic lineages occupying the Great Lakes/St Lawrence drainage basin (black bars, Atlantic Lineage) and the Upper Great Lakes/Hudson Bay/Mississippi/Nelson/McKenzie drainages (grey bars, Mississippi Lineage), respectively. The distribution of individuals in multivariate space is expressed as a percentage of individuals from each group that occupy a particular region along the canonical axis.



**Figure 4.** Canonical distribution of body shape of *Culaea inconstans* from 80 populations spanning the range of the species in multivariate space. Individual data points represent canonical group centroids for the first and second canonical functions. Grey circles, Mississippi lineage; black circles, Atlantic lineage. Numbered Mississippi lineage populations in the area of overlap correspond to map designations and demonstrate homogenous shape of populations from both lineages within the Great Lakes basin. CV, canonical variate.

for the population effect ( $N = 80$ ) and geographical positioning detected both latitudinal (Pearson  $R = 0.75$ ,  $P < 0.001$ ; Fig. 5A) and longitudinal (Pearson  $R = -0.76$ ,  $P < 0.001$ ; Fig. 5B) clinal patterns of variation when all 80 populations were pooled across the range of the species. Similar longitudinal clines (Pearson  $R = 0.55$ ,  $P < 0.004$ ) were found using the reduced number of genetically sequenced populations, as well as borderline significant latitudinal clines (Pearson  $R = 0.40$ ,  $P = 0.05$ ). In general, extreme slim-bodied and deep-bodied phenotypes were found to occur at the northwestern and south-eastern edges of the range, respectively (Fig. 5).

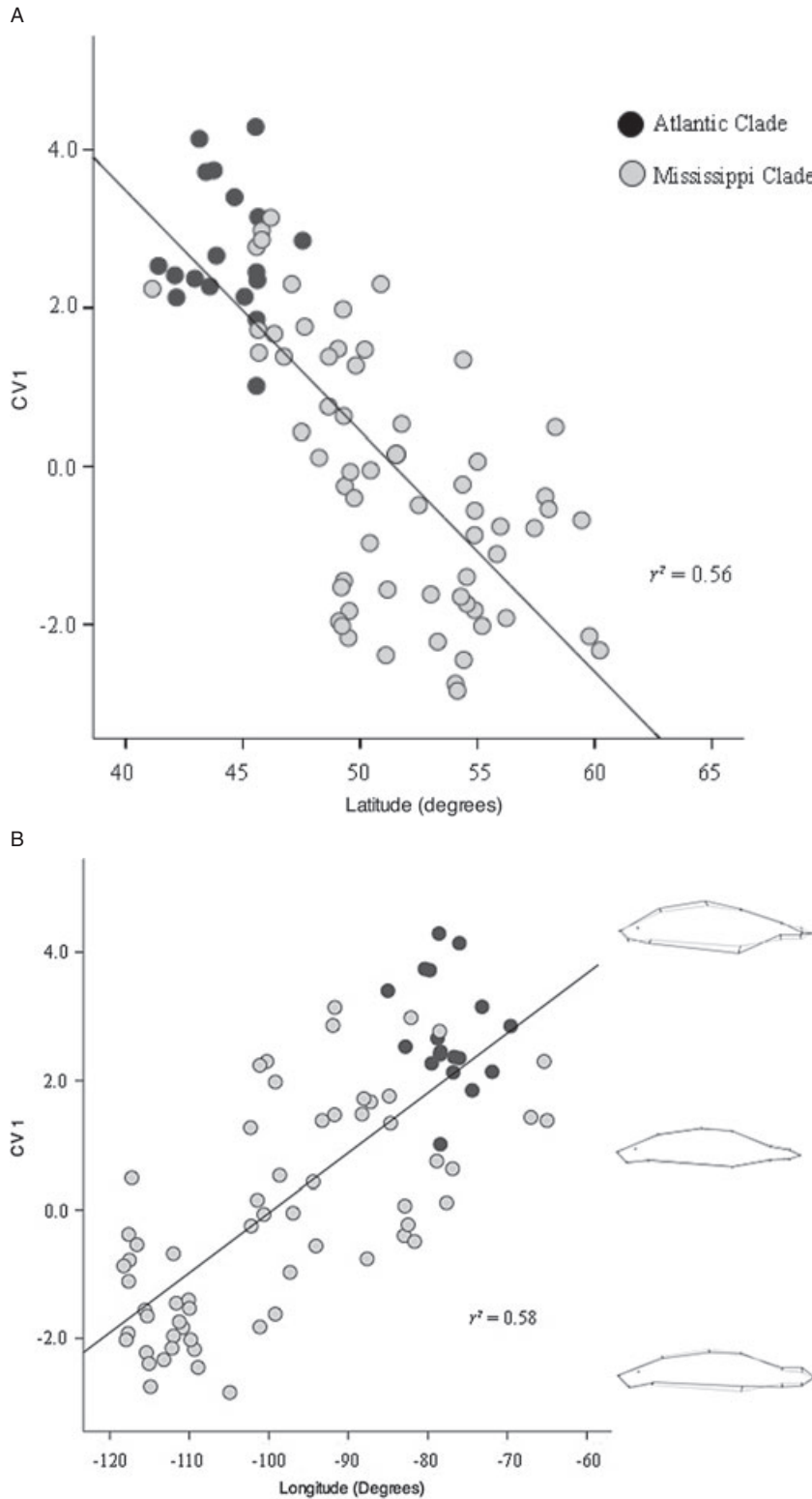
Because variation in body shape among populations showed strong clinal geographic structuring patterns, we conducted a secondary MANCOVA upon the entire dataset (80 populations) to investigate the influence of geographical differentiation on clastic patterns of shape diversity, specifying habitat type and lineage affiliation as fixed effects and centroid size, latitude, and longitude as covariates. In the primary model, we included all interactions between factors and covariates. Only three interactions (lineage  $\times$  centroid size, habitat  $\times$  latitude, and habitat  $\times$  longitude) were found to be significant. The effect sizes in all cases

were inconsequential (3–7%), so they were removed in the final model. No interaction between the main factor of interest (lineage affiliation) and either of the geographical covariates was detected.

Habitat type, and the interaction between the habitat and lineage terms, again explained relatively little of the morphological partial variation in *Culaea* (12% and 10%, respectively). By contrast, longitudinal and latitudinal gradients explained substantially more of the partial variance than that attributable to lineage affiliation (24% and 28% versus 14%; Table 2), suggesting that across the range of the species, much of the observed phenotypic differentiation between lineages can be attributed to underlying gradual geographic differentiation.

#### PATTERNS OF SIZE DIFFERENCE

In the subset analysis, Mississippi lineage fish were found to be geometrically larger than Atlantic lineage fish [analysis of variance (ANOVA):  $F = 4.22$ , d.f. = 1,  $P = 0.04$ ] but this effect disappeared when additional populations were included (ANOVA:  $F = 0.25$ , d.f. = 1,  $P = 0.62$ ). We found no evidence of a systematic size difference between *C. inconstans* collected from lake



**Figure 5.** Latitudinal (A) and longitudinal (B) morphological shape variation of *Culaea inconstans* across the species' range. Individual data points represent population centroids of the first canonical variate (CV) describing the separation of populations in multivariate shape space (black circles, Atlantic clade; grey circles, Mississippi clade). Thin-plate spline visualizations are magnified by a factor of three and depict clinal patterns of shape change with respect to the common reference shape.

**Table 2.** Results of expanded multivariate analysis of covariance (80 populations) investigating the influence of geography on variation in body shape between two clades of brook stickleback, *Culaea inconstans*, collected from both lake and river environments

Factor	<i>F</i>	d.f.	<i>P</i>	Wilks's partial $\eta^2$ (% variance explained)
Lineage	98.46	22,1318	< 0.001	14%
Habitat	85.78	22,1318	< 0.001	12%
Lineage $\times$ Habitat	72.64	22,1318	< 0.001	10%
Centroid size	31.56	22,1318	< 0.001	33%
Latitude	24.26	22,1318	< 0.001	28%
Longitude	20.44	22,1318	< 0.001	24%

*F*-values are based on Wilks' lambda, and the partial variance explained was estimated using Wilks' partial  $\eta^2$ .

and river habitats in either analysis (expanded, ANOVA:  $F = 1.63$ , d.f. = 1,  $P = 0.20$ ; subset, ANOVA:  $F = 2.63$ , d.f. = 1,  $P = 0.11$ ). Additionally, we did not detect either latitudinal (Pearson  $R = 0.08$ ,  $P = 0.50$ ) or longitudinal (Pearson  $R = -0.06$ ,  $P = 0.59$ ) clinal variation in size, irrespective of cladistic or habitat affiliation.

## DISCUSSION

The brook stickleback, *C. inconstans*, is continuously distributed from the east coast of Canada westward to British Columbia, and from the Ohio and Missouri Rivers northward to Great Slave Lake. Historically, *C. inconstans* was considered to be a single, highly variable species (the specific designation '*inconstans*' originally referred to morphological variability among dorsal spine numbers; Kirtland, 1840); however, recent phylogeographic analysis has indicated that the species was subdivided into two genetically distinct allopatric lineages prior to the last Pleistocene glaciation (Gach, 1996; Mattern, 2006). Reconstruction of refugial use and post-glacial colonization from mtDNA haplotypes suggests that *Culaea* sheltering in the mid-southern Mississippi refugium during the Pleistocene (Cross, Mayden & Stewart, 1986; Crossman & McAllister, 1986; Mandrak & Crossman, 1992; Mattern, 2006) migrated northward following glacier recession and expanded both westward and eastward through Lakes Agassiz (Falk, 1972; Rempel & Smith, 1998), Barlow and Ojibway (Gilhen, 1970; Garside & Green, 1972; Dyke & Prest, 1987; Mattern, 2006). By contrast, northern movement by fish sheltering in the south-eastern Atlantic refugium resulted in the colonization of the lower Great Lakes via the Allegheny and Susquehanna Rivers (Gach, 1996; Mattern, 2006; Stepien, Murphy & Strange, 2007). We investigated the extent and patterns of phenotypic differentiation in *Culaea* within the context of historical and environmental information, predicting that differentiation

that is primarily the result of accumulation of differences under long periods of allopatry should show congruence with cladistic patterns, whereas a consistent difference between populations found under different environmental conditions suggests alternative adaptive evolutionary processes (Thompson *et al.*, 1997; Tregenza, Pritchard & Butlin, 2000; Stayton, 2005).

The initial results obtained suggested that morphological differentiation is most consistent with historical hypotheses of refugial isolation and allopatric differentiation: typological *C. inconstans* descendents from the eastern Atlantic refugium are deep bodied, with narrow caudal peduncles and shortened, robust skulls, whereas descendents of the western Mississippi refugium are comparatively slim bodied, with deep caudal peduncles and elongated, gracile skulls). Further analysis, however, revealed strong patterns of gradual differentiation by distance, with individuals from the western extremes exhibiting the most fusiform shape and individuals from the eastern extremes exhibiting the deepest torsos. Mean cladistic differences in body shape thus appear to be an artefact of underlying clinal variation across the species range that is concordant with the distribution of the two lineages in North America. These results are consistent with previous observations made by Nelson (1969), who reported that *C. inconstans* from the western portions of the range were slimmer than available eastern specimens, and similar patterns of overlapping morphological trait values and disparity between spatial patterns of mtDNA and morphological and nuclear data have been reported for Atlantic and Mississippi refugial lineages of the banded killifish in the Great Lakes, *Fundulus diaphanous* (April & Turgeon, 2006), indicating that our results may represent a more general pattern for post-glacial fishes in the Great Lakes.

Latitudinal clines in meristic characters (e.g. number of gill rakers, fin rays and vertebrae) have

been documented for many North American fishes (Sargent *et al.*, 1984; Holcik & Nagy, 1987). Such clines are thought to represent effects of numerous environmental factors on development, with temperature, salinity, and habitat productivity foremost among them (Georgakopoulou *et al.*, 2007). The data from the present study indicate that spatially varied environmental factors might affect body shape in *Culaea* as well. In general, northern populations of *Culaea* exhibit shallower bodies and possess deeper caudal peduncles than southern populations. These patterns are consistent with a previous report of a northwest to southeast increase in the length of dorsal and pelvic spines and the degree of development of the pelvic skeleton in *C. inconstans* (Nelson, 1969; Andraso & Barron, 2002), as well as an increase in the number of dorsal spines with latitude (Hansen, 1939; Lawler, 1958).

For a variety of species, elongate bodies and deeper caudal peduncles have been linked to selection for increased fast start escape performance in response to large predators that are not gape limited (Ghalambor, Reznick & Walker, 2004; Langerhans & de Witt, 2004), whereas large (Reznick & Endler, 1982; Crowl & Covich, 1990; Reimchen, 1991), deep bodies (Bronmark & Miner, 1992) with longer spines (Kuhlmann, Kusch & Heckmann, 1999; Tollrian & Dodson, 1999) improve escape from gape-limited predators. The existence of such a generalized morphological response to alternative predation pressures in multiple taxa suggests that this interaction may have played a role in determining the shape of individuals in some of the brook stickleback populations that we censused. Brook stickleback populations, however, are often associated with a mixed predator assemblage wherein selection pressures fluctuate depending upon the relative abundance of predator species, the density of conspecifics and the availability of cover (Zimmerman, 2006, 2007). Additionally, some characters (e.g. long spines) that decrease the effectiveness of predation on adults increase predation on juveniles (Reimchen, 1980; Reist, 1980; Mikolajewski *et al.*, 2006); thus, further investigations of the correlation between North American predator distributions, body shape, and escape performance would benefit our understanding of the possible existence of such a dynamic in this species.

The strong linear correlation between longitudinal distance and shape in the absence of an identifiable environmental gradient suggests that phenotypic differentiation in *Culaea* may also have been mitigated by spatially restricted gene flow between locally available populations (Wright, 1943; Mayr, 1963; Coyne & Orr, 2004). Local gene flow, coupled with wide demographic fluctuations, has been invoked to explain an observed lack of locally adapted populations in brook

stickleback in Manitoba (Moodie, 1986), as well as clinal variation in the body shape of outlet morphs of the closely-related threespine stickleback with increasing geographic distance away from the lake (Moore & Hendry, 2005; Moore *et al.*, 2007).

We found little evidence that differences in body shape were due to major habitat associations (lake versus river/stream), unlike the situation found in *Gasterosteus aculeatus* in which distinctive river and lake morphs have evolved (see also variation in body depth between lake and stream forms of eastern North American slimy sculpins; Bookstein *et al.*, 1985). The results from the present study suggest that general adaptive evolution to alternative aquatic environments is not as widespread in *C. inconstans* as it is in Pacific coast threespines. Throughout their geographical range, *C. inconstans* preferentially inhabit shallow, heavily vegetated areas typically located around the perimeter of the water body or its outflows, regardless of whether they are in lakes, rivers, or streams (Wootton, 1976). *Gasterosteus aculeatus*, on the other hand, inhabits a wide range of environments, ranging from tidepools and estuaries, through large freshwater lakes to small acidic, tea stained creeks. Indeed, frequent reports of discrete genetic population structure associated with little or no environmentally induced diversification are generally attributed to habitat homogeneity on both regional and global scales (Taylor & Dodson, 1994; Bowen *et al.*, 2001; Colborn *et al.*, 2001; Santos *et al.*, 2006).

In summary, we found strong patterns of nonrandom phenotypic structuring across the species range in *C. inconstans* with the most extreme phenotypes occurring at the northwestern and southeastern edges of the range. However, the patterns of morphological differentiation are not consistent with either discontinuous lineage-affiliated morphotypes or a repeated adaptive response to alternative environments. Phenotypic differentiation shows strong clinal variation, suggesting that any historical effects of isolation upon body shape differentiation have been mitigated by the influence of relatively recent local effects, such as continually varying natural selection across North America to environmental factors, including temperature and/or predation regime, and/or potential local gene flow between populations, and that these factors are responsible for most of the phenotypic differentiation in this widespread fish.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Locality information and lineage and habitat assignment for wild-caught brook stickleback, *Culaea inconstans*, collected from 80 sites across Canada and the United States ( $N = 1420$ ). Atlantic lineage (River):  $N = 218$ ; Atlantic lineage (Lake):  $N = 68$ ; Mississippi lineage (River):  $N = 643$ ; Mississippi lineage (Lake):  $N = 491$ . Map location numbers indicate placement in Fig. 1. Asterisks indicate genetically sequenced populations used in subset analysis.

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