Genetic coupling of female mate choice with polygenic ecological divergence facilitates stickleback speciation

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SUMMARY

Ecological speciation with gene flow is widespread in nature [1], but presents a conundrum: how are associations between traits under divergent natural selection and traits that contribute to assortative mating maintained? Theoretical models suggest that genetic mechanisms inhibiting free recombination between loci underlying these two types of traits (hereafter, "genetic coupling") can facilitate speciation [2-4]. Here, we perform a direct test for genetic coupling by mapping both divergent traits and female mate choice in a classic model of ecological speciation: sympatric benthic and limnetic threespine stickleback (*Gasterosteus aculeatus*). By measuring mate choice in F2 hybrid females, we allowed for recombination between loci underlying assortative mating and those under divergent ecological selection. In semi-natural mating arenas in which females had access to both benthic and limnetic males, we found that F2 females mated with males similar to themselves in body size and shape. In addition, we found two quantitative trait loci (QTL) associated with female mate choice that also predicted female morphology along the benthic-limnetic trait axis. Furthermore, a polygenic genetic model that explains adaptation to contrasting benthic and limnetic feeding niches [5] also predicted F2 female mate choice. Together, these results provide empirical evidence that genetic coupling of assortative mating with traits under divergent ecological selection helps maintain species in the face of gene flow, despite a polygenic basis for adaptation to divergent environments.

RESULTS

We tested for genetic coupling between loci underlying ecologically divergent traits and assortative mating by examining morphological and genomic determinants of female mate

choice in a sympatric pair of benthic and limnetic threespine stickleback from Paxton Lake in British Columbia, Canada. Species pairs of stickleback have evolved repeatedly in multiple postglacial lakes in British Columbia [6,7]. Each lake contains a larger, deeper bodied benthic form that inhabits inshore habitats, and a smaller, shallow bodied limnetic form that inhabits open water [8,9]. These species are morphologically adapted to their contrasting food sources: benthic stickleback primarily feed on invertebrates inhabiting the substrate or attached to vegetation, whereas limnetics specialize on zooplankton [10-12]. Although hybrids exist in the wild [13-15] and there are no strong intrinsic incompatibilities [14,16], benthics and limnetics show nearly complete assortative mating in experimental trials [17]. Previous no-choice mating trials suggested that benthic and limnetic females prefer mates with similar body size [18-20] and shape [20]. In this study, we conducted female mate choice experiments in ponds that allowed females to access both benthic and limnetic males in habitats that closely mimic those found in the wild [5]. By examining whether recombinant F2 hybrid females that vary in phenotype mate with benthic or limnetic males, we tested whether females prefer to mate with individuals that have similar phenotypes to themselves. We also identified QTL for female mate choice and morphology to test whether genomic regions associated with mate choice correspond to regions determining phenotypic traits under divergent selection. These represent the first direct tests of genetic coupling in this vertebrate system.

F2 females prefer males with a similar body shape and size

Body shape of F2 hybrid females was positively associated with the shape of chosen mates. We defined shape based on 17 external morphological landmarks (34 x- and y-coordinates;

Figure S1), with landmarks for each fish rotated and scaled to the same centroid size. We used principal component (PC) analysis of the morphological landmark coordinates to summarize continuous variation in phenotypes of pure-species males and F2 females, which allowed us to examine shape variation associated with female choice within and between benthic and limnetic males. The first PC axis (PC1) separated male from female fish and was not analyzed further. PC2 separated benthic males from limnetic males, with F2 females intermediate (Figure 1A). Females that mated with limnetic males had lower (more limnetic-like) PC2 shape values than those that mated with benthic males (χ^2=17.46; P=2.9×10^{-5}: partial R^2=0.072; Figure 1B). Remarkably, among F2 females that mated with benthic males, those most benthic-like in shape tended to mate with benthic males that were closer to the benthic extreme of the PC2 shape distribution ($F_{1,444}$=6.65; P=0.01; partial R^2=0.015; Figure 1C). We did not detect a similar trend in F2 females that mated with limnetic males ($F_{1,444}$=0.33; P=0.56; partial R^2=0.0008; Figure 1C). We also analyzed F2 female shape using a discriminant function that separates benthic from limnetic males based on the 34 external morphological landmark coordinates (Table S1). In accordance with the results above using PC2, females that mated with benthic males had a more benthic-like shape than those that mated with limnetic males (χ^2=16.23; P=5.6×10^{-5}; partial R^2=0.065). When centroid size was used as a covariate in these analyses, the correlations between the shape of F2 hybrid females and the chosen males remained (data not shown), suggesting that body shape is an important component of female mate choice.

Body size of hybrid F2 females also predicted mate choice. Females with larger centroid sizes preferentially mated with males of the larger, benthic species (χ^2=17.79; P=2.5×10^{-5};

partial R^2=0.103; Figure 1D). Among F2 females that mated with benthic males, there was a non-significant tendency for the largest of them to mate with the largest benthic males ($F_{1,444}$=1.89; P=0.17; partial R^2=0.004; Figure 1E). There was a similar positive tendency among F2 females that mated with limnetic males, though again this pattern was not significant ($F_{1,444}$=1.06; P=0.30; partial R^2=0.002; Figure 1E).

Eight of 34 shape traits (x- and y-coordinates of 17 morphological landmarks; Figure S1), found mainly in the head and the caudal region of F2 females, were significantly associated with mate choice when tested one at a time: y1, x2, y2, x5, y6, y9, y15, and y16 (FDR-adjusted P<0.05). The importance of some of these traits to mate choice is also indicated by their contribution to scaling on the benthic-limnetic discriminant function and their loading on PC2 (Table S1). The scaling values of two jaw coordinates (y1 and y2) are within the top five scaling values on the first linear discriminant axis. Both of those coordinates, along with coordinates at the insertion of the dorsal (y15) and anal (y16) fins, were also within the top five loadings on PC2.

Genetic coupling of mate choice and ecological traits

We found two QTL peaks for F2 female mate choice (Figure 2A; Table S2), on chromosomes 14 (LOD= 4.5, PVE=7.52) and 21 (LOD=4.61, PVE=10.07). For the QTL on chromosome 14, F2 females homozygous for the benthic allele (BB) were more likely to choose a benthic mate than either the limnetic homozygotes (LL) or heterozygotes (LB) (Figure 3; means: LL=0.64, LB=0.71, BB=0.86, where 0 and 1 indicate limnetic and benthic mate choice, respectively). The QTL on chromosome 21 showed a different pattern, where the

heterozygote was more likely to choose a benthic mate than either homozygote (means: LL=0.58, LB=0.83, BB=0.61).

F2 female mate choice is associated with her own shape and size, despite the opportunity for recombination between loci underlying the traits, suggesting either pleiotropy or physical linkage between morphology and mate choice loci. For this reason, we also investigated the genetic architecture of F2 female morphology. The results suggest that the genetic basis of morphological traits correlated with mate choice is more widely distributed across the genome than implied by the two QTL we identified for mate choice. Of the QTL for body size and the three measures of shape variation predicting mate choice (i.e. eight x- and y-landmark coordinates, PC2, and the benthic-limnetic linear discriminant function), a single QTL for body shape overlaps with a mate choice QTL (Table S2). We found a single QTL on chromosome 9 for centroid size (Figure 2C; LOD=6.97, PVE=10.08) and two QTL for PC2: one on chromosome 4 (LOD=4.04; PVE=5.97) and one on chromosome 7 (LOD=6.67; PVE=9.71). Of the eight landmark traits correlated with mate choice, five were influenced by QTL distributed across five chromosomes (Table S2). One of these QTL, for a jaw landmark coordinate (y2), overlapped with the QTL for PC2 on chromosome 4. Finally, two QTL were associated with the discriminant function separating benthic and limnetic morphology (Figure 2B). One of the QTL overlapped with the mate choice QTL on chromosome 14 (LOD=4.23; PVE=6.24), and the other mapped to chromosome 12 (LOD=4.83; PVE=7.10). At both QTL, the benthic allele was associated with a higher (more benthic-like) value of the morphological trait (Table S2).

Despite this distributed genetic architecture for F2 female body size and shape, two lines of evidence suggest genetic coupling between the QTL detected for mate choice and those detected for ecologically divergent traits. First, a linear model containing the two QTL detected for mate choice on chromosomes 14 and 21 explained a significant amount of variation in the benthic-limnetic discriminant function (Figure 3; P=0.015; LOD=2.73; PVE=4.08). Second, an additive, polygenic QTL model that predicted F2 hybrid position along the benthic-limnetic ecological niche axis provided by an earlier study of the same species pair [5] also accounted for a significant proportion of the variance in mate choice in the current study (P=0.001; LOD=10.48; PVE=21.4). The linear model based on these QTL genotypes also explained a significant proportion of the variance in the benthic-limnetic discriminant function in our experiment (P=0.00005; LOD=13.24; PVE=18.29).

DISCUSSION

The genetic basis of mate choice has consequences for the efficacy of ecological speciation with gene flow. We used data on associations between morphology, genetics, and mate choice to test predictions of the "genetic coupling" model for the evolution of mate choice. We investigated the genetic basis of interspecific mate choice in a sympatric species pair of stickleback that continue to undergo a low level of hybridization in the wild [13-15]. By measuring mate choice in F2 hybrids, which allowed the opportunity for some recombination between loci encoding mate choice and those encoding traits under divergent selection, we found strong evidence for genetic coupling. First, we found that F2 hybrid females mated with males that were more similar to themselves in shape and size. This result implies that assortative mating between like phenotypes was not eliminated by

recombination in this hybrid population. Second, we found two QTL for mate choice that also explained variation in body shape. Finally, we found that a QTL model that explained variation in F2 hybrid niche use along the benthic-limnetic axis in a previous study [5] also explained variation in both F2 female shape and mate choice in our study. Together, these results are consistent with genetic coupling for the evolution and maintenance of assortative mating in this stickleback species pair.

The absence of free recombination between loci for mate choice and loci for traits under divergent selection (i.e. genetic coupling) could be due to either pleiotropy or close linkage. Felsenstein [2] showed that both mechanisms increase the likelihood of speciation and species persistence in the face of gene flow. Pleiotropy can result from phenotype matching, whereby individuals in both species (and their hybrids) prefer to mate with individuals having a similar phenotype to their own. This corresponds to Felsenstein's "one-allele" model for the evolution of mate choice, because at a given mating locus the same allele encodes conspecific preference in both species (e.g., it encodes a phenotype matching behavior "mate with like"). When the phenotype matching alleles are fixed in both species, the observed genetic determinants of variation in mate choice are the allelic variants at the loci underlying traits upon which matching is based. This contrasts with Felsenstein's "two-allele" model with linkage, in which distinct alleles controlling assortative mating between alternative phenotypes are physically linked to genes for traits under divergent natural selection.

184 By themselves, our results do not allow us to distinguish between genetic coupling caused
185 by phenotype matching (one-allele model) and genetic coupling caused by physical linkage
186 between alleles for mate choice and traits (two-allele model with linkage), because in both
187 cases mate choice in recombinant hybrids should map to the regions of the genome
188 responsible for variation in phenotypic traits. However, previous studies in this system are
189 most consistent with a one-allele mechanism. In no-choice mating trials between
190 heterospecifics, females mate with males that are similar in size and shape to themselves
191 [18-20]. Importantly, non-genetic manipulation of the sizes of females changes the size of
192 males with which they prefer to mate [19]. This result is strong evidence for the one-allele
193 phenotype matching mechanism, at least for body size, because this non-genetic
194 phenotypic manipulation of female body size yields no change in genes for body size
195 preference, even if linked to genes for body size [19]. However, longer-term studies with
196 more advanced generation hybrids to break down potential linkage between the loci that
197 underlie body size and shape and the loci that underlie mate preferences are needed to
198 provide more direct evidence that a one-allele mechanism contributes to genetic coupling
199 of traits under divergent selection and mate choice in this system.
200
201 The proximate mechanism for phenotype matching suggested by our data and
202 demonstrated by other studies is not clear [19]. How do female fish perceive and match
203 subtle variations in their own shape and size to that of their mate? Proposed mechanisms
204 often include sexual imprinting or social learning. A few studies have found evidence for
205 sexual imprinting in mate preference between stickleback species [21,22]. Yet, all F2
206 females used in our study were produced by natural mating between F1 hybrid parents,

which possess a much lower amount of size and shape variation than is seen between the two parent species, thus reducing the opportunity for imprinting or learning. It is possible to imagine that during courtship a female would be capable of evaluating her own body size relative to that of a male, but it seems far less plausible that she would be able to compare subtle differences in their body shapes. Instead, phenotype matching might occur not by direct comparison of morphology but rather by a shared feeding habitat preference between individuals that are similar in morphology. In threespine stickleback, size and shape is strongly associated with niche use both among species and among F2 hybrid individuals varying in morphology [5,10-12]. For example, the most benthic-like F2 females might feed preferentially in the same pond regions as do male benthics, and this higher encounter rate between like individuals might then lead to a higher probability of mating.

Regardless of the underlying mechanism, our results provide empirical evidence that genetic coupling is important for the persistence of species in the face of gene flow. Although genetic coupling, either via a one-allele mechanism [23] or a two-allele mechanism with linkage, has now been shown in a few other systems, in all of these cases the divergent traits are encoded by one or a few loci of relatively large effect [24-30]. However, such a simple genetic architecture for traits under divergent selection might be relatively rare. Our previous studies in stickleback have indeed shown that the genetic architecture of adaptation in this system is highly polygenic [5,31,32]. This diffuse genetic architecture of adaptation makes a two-allele model with tight linkage seem less plausible, because this would require a large number of mate choice alleles to be distributed across the genome, all in tight linkage with alleles for traits under divergent selection. Under

either model, our results suggest that even when the underlying genetic architecture of phenotypes under divergent selection is polygenic and distributed across the genome, genetic coupling with assortative mating will contribute to the persistence of species in the face of gene flow.

AUTHOR CONTRIBUTIONS

Conceptualization: M.E.A., G.L.C., D.S., and C.L.P.; Methodology: M.E.A., G.L.C., D.S., and C.L.P.; Formal Analysis: R.A.B., M.E.A., G.L.C., D.S., and C.L.P.; Investigation: M.E.A., G.L.C., J.B., N.L.B., S.R.M., and M.E.D.; Resources: Y.F.C., F.C.J., and D.M.K.; Writing – Original Draft: R.A.B., G.L.C., D.S., and C.L.P.; Writing – Review & Editing: – M.E.A., N.L.B., Y.F.C., and D.M.K.; Supervision: M.E.A., D.S., and C.L.P.; Funding Acquisition: M.E.A., D.S., and C.L.P.

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FIGURE LEGENDS

Figure 1. Mate choice of F2 females is associated with shape and centroid size of males and females. Shape is summarized using principal component analysis of 17 landmarks. (A) PC2 separates benthic and limnetic males, with F2 females intermediate. In F2 females, PC2 is significantly associated with the male species chosen (B) and with variation in male PC2 scores when benthic males were chosen (C). In F2 females, centroid size is significantly associated with mate choice (D), but not with variation in male centroid size when benthic males were chosen or when limnetic males were chosen (E). See also Figure S1, Table S1, Table S3.

Figure 2. QTL mapping of female mate choice, body shape, and body size. The graphs show LOD scores across the 21 stickleback chromosomes for: (A) female mate choice, (B) benthic-limnetic discriminant function, and (C) centroid size. Dotted lines: α=0.1 genome-wide significance cutoff based on 10,000 permutations. See also Table S2, Table S3, Table S4.

Figure 3. Effects of two QTL on female mate choice and body shape. The effects of the mate choice QTL on chromosome 14 (A,C) and 21 (B,D) are shown for mate choice (A,B) and shape, represented by discriminant function score (C, D). QTL for mate choice is based on a binary response variable with 0=limnetic and 1=benthic. Points represent mean for each female genotype and error bars indicate 95% confidence intervals. See also Table S2.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Catherine Peichel (catherine.peichel@iee.unibe.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal experiments involved threespine stickleback (*Gasterosteus aculeatus*) fish and were approved by the University of British Columbia Animal Care Committee (protocols A07-0293, A11-0402) and the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol 1797).

Generation of F2 females

In 2007, we used wild-caught adult fish to make six *in vitro* interspecific crosses. Three crosses involved a limnetic female and three crosses involved a benthic female. We stored their bodies in 95% ethanol for DNA analysis. We reared the resulting F1 hybrids in the laboratory. In March 2008, F1 hybrids were introduced to two outdoor experimental ponds on the campus of University of British Columbia (described in [5]). For the first, we randomly selected 24 F1 hybrid adults from a cross involving a limnetic female, and 24 F1 hybrid adults from a cross involving a benthic female. We took a sample of caudal fin tissue from each individual F1 hybrid for DNA analysis and then released them into two separate mesh enclosures within a pond. The enclosures were designed to allow only full-sib matings between F1s and to allow F2 hybrid offspring to escape the enclosure into the

pond. However, we realized that the enclosures were limiting the number of F2 hybrids that were produced. Thus, we established a second rearing pond, for which we randomly selected five F1 hybrids of each sex from the remaining four crosses. We took a sample of caudal fin tissue from each individual for DNA analysis and then released them into the pond. This design allowed interbreeding between F1s from different crosses. In 2009, we used wild-caught adult fish to make two additional *in vitro* interspecific crosses. One cross involved a limnetic female and the other a benthic female. We stored their bodies in 95% ethanol for DNA analysis. We reared the resulting F1 hybrids in the laboratory. In May 2010, we initiated two F2 rearing ponds to increase the number of F2 hybrids generated and to allow only full-sib matings between F1 hybrids. We randomly selected 35 F1 hybrid adults from the cross involving a limnetic female and 35 F1 hybrid adults from the cross involving a benthic female. We took a sample of caudal fin tissue from each individual F1 hybrid for DNA analysis and then released them into their respective ponds. After release, the F1 hybrids were allowed to mate freely with their full-siblings in the same pond throughout the breeding season. For an overview of the source and numbers of the F2 females used in these experiments in both years, see Figure S2.

The ponds (25 x 15 m surface area) contained a sloping shallow zone and a deep open-water zone (6 m deep), thereby providing feeding and nesting habitat for both species [5]. In each spring of 2007 – 2010, we inoculated the ponds with macrophytes, sediments and water full of aquatic insects, mollusks and plankton from Paxton Lake. Each time we added 1.25kg of a 25.5:1 mix of 50% pure KNO_3 : KH_2PO_4 to stimulate primary production.

METHOD DETAILS

F2 female mate choice experiment in ponds

We established three 'mating arena' ponds during the study (Figure S2), one in the summer of 2009 and two in the summer of 2011 to increase the area available for males to establish territories. On April 20 and 21, 2009, we added 122 wild-caught limnetic males and 117 wild-caught benthic males to the mating arena pond. From April 22 to June 1, 2009, we used minnow traps to catch 331 gravid F2 females from the two rearing ponds initiated the previous year and transferred them to the mating arena. On April 28 and 29 2011, we added 64 wild-caught limnetic males and 61 wild-caught benthic males to mating arena 1, and 64 wild-caught limnetic males and 62 wild-caught benthic males to mating arena 2. From May 2 to June 23, 2011, we used minnow traps to catch gravid F2 females from the rearing ponds initiated the previous year and transferred 219 F2 females to mating arena 1 and 218 F2 females to mating arena 2. We photographed all fish on their left side and took a sample of caudal fin tissue for DNA analysis before releasing fish into mating arenas.

From April 30 to July 17, 2009 and May 17 to July 14, 2011, we used snorkeling and SNUBA (Surface Nexus Underwater Breathing Apparatus) gear in each mating arena pond once every 3-4 days (2009) or once per week (2011) to collect fertilized eggs from male's nests. Upon collection, eggs were inspected for their extent of development. If eyes were visible, the entire clutch was stored directly in 95% ethanol for DNA parentage analysis. If eyes were not yet visible, the clutch was split approximately in half. One half was stored directly in 95% ethanol and the other half was incubated in an aquarium to allow further development to ensure enough DNA for parentage analysis before being stored in 95%

ethanol. When multiple clutches were found within the same nest (determined visually via different egg clumps and extent of egg development), each clutch was treated separately.

Parentage assignment

For the mate choice experiment conducted in 2009, we genotyped 331 F2 females, 117 benthic males, 122 limnetic males, and 245 fertilized eggs (1 per clutch) or free-swimming juveniles with 18 microsatellite markers (Table S3) following [33]. For the mate choice experiment conducted in 2011, we genotyped 437 F2 females (219 in arena 1, 218 in arena 2), 123 benthic males (61 in arena 1, 62 in arena 2), 128 limnetic males (64 in arena 1, 64 in arena 2), and 328 fertilized eggs (1 per clutch) or free-swimming juveniles (186 in arena 1, 142 in arena 2) with 19 microsatellite markers (Table S3). Parentage was assigned using the R package 'MasterBayes' [34] with the following parameters: E1=0.01, E2=0.01, mm.tol=10 (DRYAD data file 'pedigree.all.csv').

In total, 383 unique F2 females were identified in the parentage analyses (Figure S2). However, for further analyses, we only considered the 467 unique mating events for which the probability of parentage assignment of a fertilized egg or free-swimming juvenile was greater than 0.75. Using these assignments, we assessed mate choice for 291 unique F2 females, of which 255 mated exclusively with a single male species while the remaining 36 chose males of the two species for separate clutches (DRYAD data file 'choice.all.csv'). Of the 255 F2 females that mated with only one species, 191 mated once, while 64 mated multiple times including one F2 female that mated with benthic males ten times.

Morphological analysis

We used 17 morphological landmarks to summarize morphology in wild-caught benthic and limnetic males and F2 females (Figure S1; DRYAD data file 'phenotypes.all.csv'). Using digital images taken of live fish alongside a ruler for scale, we recorded the x- and y-coordinates of each landmark and scaled the values using 'tpsDig' v2.12 [35]. Coordinates were superimposed, and scaled values as well as centroid sizes were calculated using Generalized Procrustes Analysis in the R package 'shapes' [36]. We summarized these landmarks using principal component analysis with the 'prcomp' function in R [37]. Custom R scripts ('Morphology.R' and 'landmarks.R') for these analyses are provided on DRYAD (http://dx.doi.org/10.5061/dryad.bs7sg).

Association of F2 mate choice and morphology

We tested for associations between mate choice and morphology of the 255 F2 females that mated with only a single male species using centroid size as a measure of body size and three measures of F2 female shape based on landmarks: (1) principal component analysis, (2) discriminant function analysis; (3) individual x and y coordinates of landmarks. For centroid size, we tested associations between F2 female size and female mate choice using a binomial generalized linear model with experimental pond as a covariate.

Principal component analysis

We used principal component analysis to examine morphological variation within F2 females as well as within and between benthic and limnetic males. A single principal component axis (PC2) separated benthic and limnetic males, with F2 females intermediate.

We used this benthic-limnetic PC axis to test associations between female morphology and female mate choice. Parents of each egg clutch, as determined from the parentage analysis, were used to determine the species of male chosen by each F2 female. We used a binomial generalized linear model to test associations between female mate choice (benthic or limnetic) and her score along the benthic-limnetic PC axis, with experimental pond as a covariate and significance assessed using the drop1 function in R. We also used linear models to compare female PC scores with the PC scores of the chosen males, with experimental pond as a covariate and mother as a random effect. Coefficients of partial determination (partial R^2) were calculated using the 'rsq' package in R [37]. We repeated these analyses using centroid size as a measure of body size in place of the benthic-limnetic PC axis.

Discriminant function analysis

We used discriminant function analysis to summarize F2 female shape morphology along a benthic-limnetic axis. We used morphological landmarks from wild-caught benthic and limnetic males to build a discriminant function with the R package 'MASS' [38]. This model had 99.8% classification accuracy using 10-fold cross-validation; only a single individual male was incorrectly classified. The model was used to predict discriminant function values for F2 females based on the same morphological landmarks. We then tested for association between this benthic-limnetic discriminant function value and mate choice in F2 females using a binomial generalized linear model with experimental pond as a covariate.

Individual x- and y-coordinates

519 To identify specific morphological landmarks that are most strongly correlated with female
520 mate choice, we also tested for associations between female mate choice and body shape
521 landmarks of F2 female phenotype. For this, we used the scaled x- and y-landmark
522 coordinates for F2 females and tested associations with female mate choice using a
523 binomial generalized linear model with experimental pond as a covariate.
524
525 **Genotyping F2 females**
526 We isolated genomic DNA from caudal fin tissue of the 16 F0 progenitors, 158 F1 hybrids,
527 and the 383 F2 hybrid females identified in the parentage analyses using Proteinase K
528 digestion, phenol-chloroform extraction, ethanol precipitation and re-suspension of the
529 precipitated DNA in 30 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). We genotyped all
530 F0, F1, and F2 individuals using Illumina's GoldenGate assay and a custom multiplex
531 oligonucleotide pool developed for a previously published collection of single nucleotide
532 polymorphisms (SNPs; [7]; Table S4). We found 494 of these SNPs to be polymorphic in at
533 least one of our crosses. The Illumina Sentrix Array Matrices used for genotyping were
534 processed at the Genomics Shared Resource of the Fred Hutchinson Cancer Research
535 Center (Seattle, WA, USA). We scored genotypes from the raw data using GenomeStudio
536 software (Illumina Inc.).
537
538 **Linkage map construction**
539 To build a linkage map, we started with the 383 genotyped F2 females in this experiment
540 (Figure S2), along with 1,348 F2 individuals from the same crosses but used in another
541 experiment [39]. Following [5], we only used F2 individuals that could be assigned to an F1

× F1 family having at least 10 full-siblings for linkage map construction and subsequent QTL analyses, resulting in the inclusion of 302 F2 females from this experiment. We first calculated pairwise recombination frequencies for each F1 × F1 family using JoinMap ver 3.0 [40]; recombination frequencies were concatenated and imported into JoinMap to produce a single linkage map. We found 21 linkage groups, which were assigned to the 21 chromosomes from the stickleback genome assembly using known SNP locations.

QTL analysis

All QTL analysis was performed in the 'R/qtl' package [41], and a custom R script 'QTL.R' is provided on DRYAD (http://dx.doi.org/10.5061/dryad.bs7sg). Although power to detect QTL of small effect is increased by having more individuals, power to detect QTL at all is reduced if the phenotypic analysis is not robust. To map mate choice, we therefore conservatively used the 200 F2 females that were: (1) included in the linkage map construction; (2) had a parentage assignment probability greater than 0.75; and (3) mated with only a single species of male (DRYAD data files: 'purechoice.gen.csv' and 'purechoice.pheno.csv'). We used the 'scanone' command with Haley-Knott regression and a binary response variable (1 = chose benthic; 0 = chose limnetic), with both family and experimental pond as covariates. To determine significance, we used 10,000 permutations and a genome-wide cutoff of α=0.1. We used this lenient threshold because our main goal was to determine whether QTL for mate choice and morphology lie in the same regions, so false positives were less of a concern than missing QTL.

564 To increase our power to detect QTL for morphological traits associated with mate choice,
565 we included all 302 F2 females used in the linkage map construction (DRYAD data files:
566 'all.gen.csv' and 'all.pheno.csv'). We conducted a similar analyses as above to find QTL for
567 centroid size as well as for our three shape measurements: 1) the PC axis that
568 differentiated benthic and limnetic shapes; 2) the benthic-limnetic discriminant function;
569 and 3) x and y coordinates of morphological landmarks. For these, we assumed a Gaussian
570 distribution for the response variable. For each significant QTL, we calculated percent
571 variance explained (PVE) under a single QTL model using the function $PVE=1-10^{(-2*LOD/n)}$
572 [41]. All shape QTL remained significant even after using centroid size as a covariate in the
573 analyses (data not shown).
574
575 Additionally, we used 'fitqtl' to investigate whether QTL peaks for mate choice could also
576 explain the predicted benthic-limnetic discriminant function values of the 200 F2 females
577 used to map mate choice. We calculated significance (χ^2 test), log odds ratio (LOD), and PVE
578 as above.
579
580 Arnegard et al. [5] defined an additive model of 11 QTL loci and significant interactions that
581 predicted F2 phenotype along the benthic-limnetic niche axis. Because the same SNP assay
582 was used here as in Arnegard et al. [5], we were able to use the same markers to test
583 whether this model could explain both morphology and mate choice in our experiment. We
584 used 'fitqtl' to compare the sum of squares of a model with pond and family covariates only
585 to a model that also included genotypes at the 11 markers identified by Arnegard et al. [5]

to explain the predicted benthic-limnetic discriminant function value as well as mate choice in the 200 F2 females used to map mate choice.

QUANTIFICATION AND STATISTICAL ANALYSIS

All analysis was conducted in R [37]. Statistical tests and software used are described in Method Details (above).

DATA AND SOFTWARE AVAILABILITY

All data files and custom R scripts required to recreate these analyses are available on DRYAD: http://dx.doi.org/10.5061/dryad.bs7sg.

SUPPLEMENTAL INFORMATION

Supplemental Information PDF contains 2 figures and 3 tables.

Table S4. Names and locations of SNPs used for linkage mapping and QTL analysis. Related to Figure 2. The positions in bp refer to the original threespine stickleback genome assembly (Broad S1, Feb. 2006; http://www.ensembl.org/Gasterosteus_aculeatus/Info/Index).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Critical Commercial Assays		
Deposited Data		
Data file ‘pedigree.all.csv’: parentage assignments for all genotyped offspring	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Data file ‘choice.all.csv’: mate choice data for 291 F2 females with parentage assignment > 0.75	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Data file ‘phenotypes.all.csv’: raw X and Y values for 17 morphological landmarks in all F2 females, wild benthic males and wild limnetic males	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Data file ‘purechoice.gen.csv’: SNP genotypes for input to Rqtl for 200 F2 females with pure mate choice	This paper	http://dx.doi.org/10.5061/dryad.bs7sg

Data file ‘purechoice.pheno.csv’: scaled morphological landmarks, size, and mate choice values for 200 F2 females with pure mate choice in Rqtl format	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Data file ‘all.gen.csv’: SNP genotypes for input to Rqtl for 302 F2 females	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Data file ‘all.pheno.csv’: scaled morphological landmarks, size, and discriminant function values for 302 F2 females in Rqtl format	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Threespine stickleback (*Gasterosteus aculeatus*) benthic x limnetic F2 females	This paper	N/A
Threespine stickleback (*Gasterosteus aculeatus*) wild benthic males	This paper	N/A
Threespine stickleback (*Gasterosteus aculeatus*) wild limentic males	This paper	N/A
Oligonucleotides		
Primers for parentage analysis	This paper, [33]	Table S3
Single nucleotide polymorphism arrays	This paper, [7]	Table S4
Recombinant DNA		
Software and Algorithms		
R Package ‘MasterBayes’	[34]	https://cran.r-project.org/web/packages/MasterBayes/MasterBayes.pdf
tpsDig v2.12	[35]	http://life.bio.sunysb.edu/ee/rohlf/software.html

R package ‘shapes’	[36]	https://www.maths.nottingham.ac.uk/personal/ild/shapes/
R core team	[37]	https://www.r-project.org/foundation/
R package ‘MASS’	[38]	https://cran.r-project.org/web/packages/MASS/MASS.pdf
GenomeStudio	Illumina	https://support.illumina.com/array/array_software/genomestudio/downloads.html
JoinMap 3.0	[40]	https://www.kyazma.nl/index.php/JoinMap/
R package ‘R/qtl’	[41]	http://www.rqtl.org/
R script ‘Morphology.R’: custom R script for statistical analyses and visualization of morphological data	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
R script ‘Landmarks.R’: functions used in Morphology.R for scaling	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
R script ‘QTL.R’: custom R script for plotting and identifying QTL for mate choice and morphology	This paper	http://dx.doi.org/10.5061/dryad.bs7sg
Other		

Figure 1

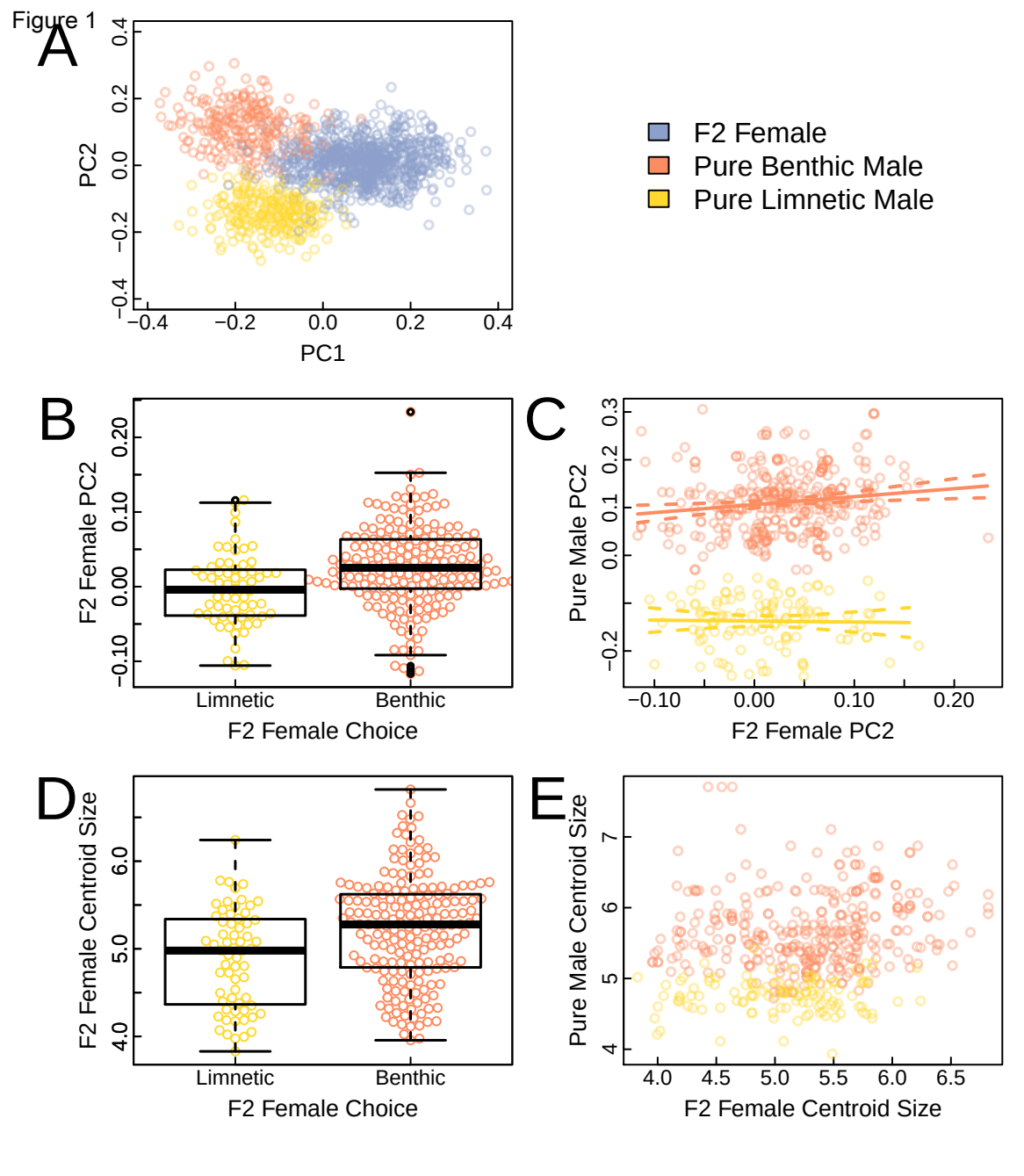

A
PC2
PC1
−0.4
−0.2
0.0
0.2
0.4
F2 Female
Pure Benthic Male
Pure Limnetic Male
B
F2 Female PC2
−0.10
0.00
0.10
0.20
Limnetic
Benthic
F2 Female Choice
C
Pure Male PC2
−0.2
0.0
0.1
0.2
0.3
−0.10
0.00
0.10
0.20
F2 Female PC2
D
F2 Female Centroid Size
4.0
5.0
6.0
Limnetic
Benthic
F2 Female Choice
E
Pure Male Centroid Size
4
5
6
7
4.0
4.5
5.0
5.5
6.0
6.5
F2 Female Centroid Size

Figure 2

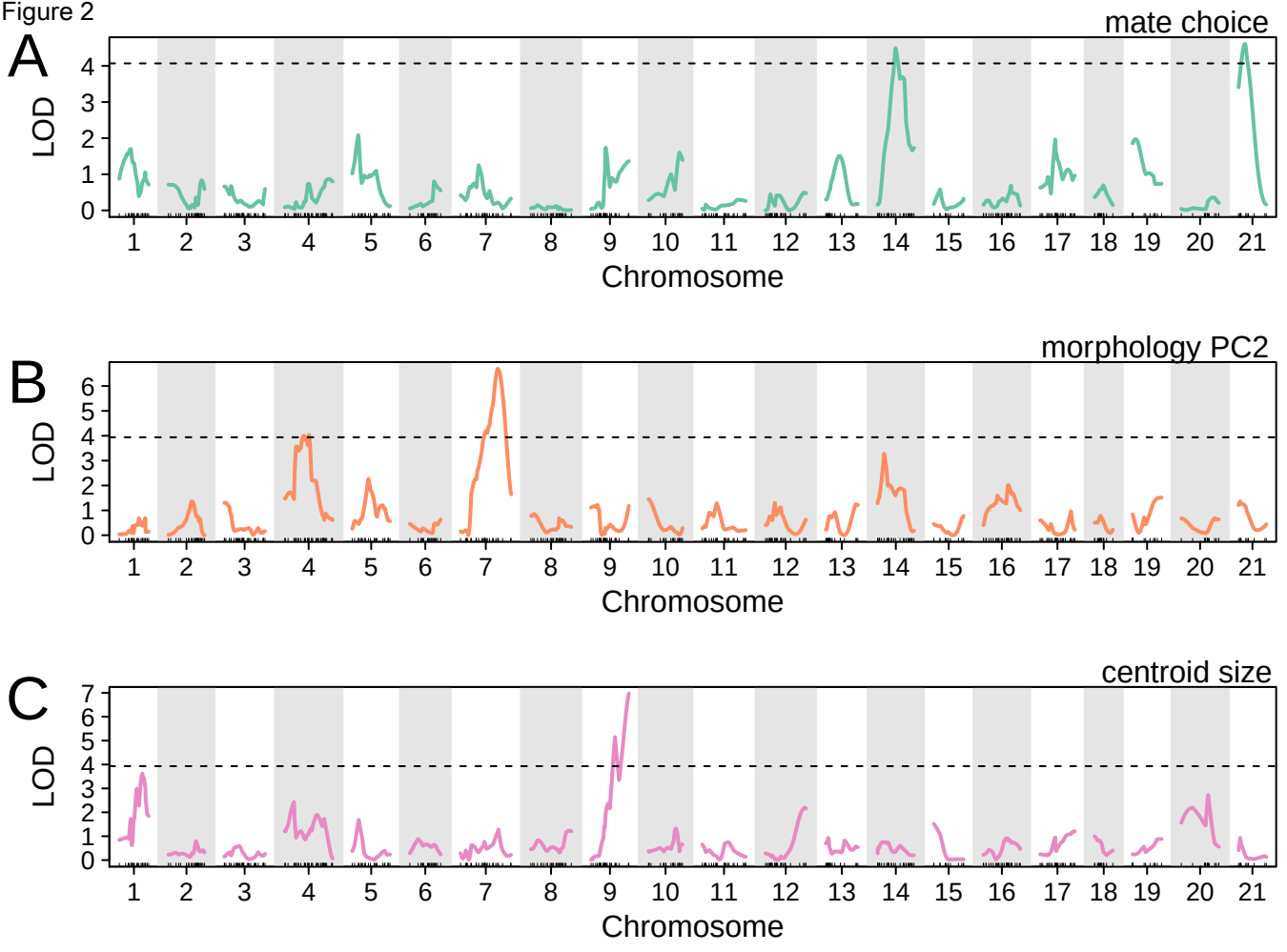
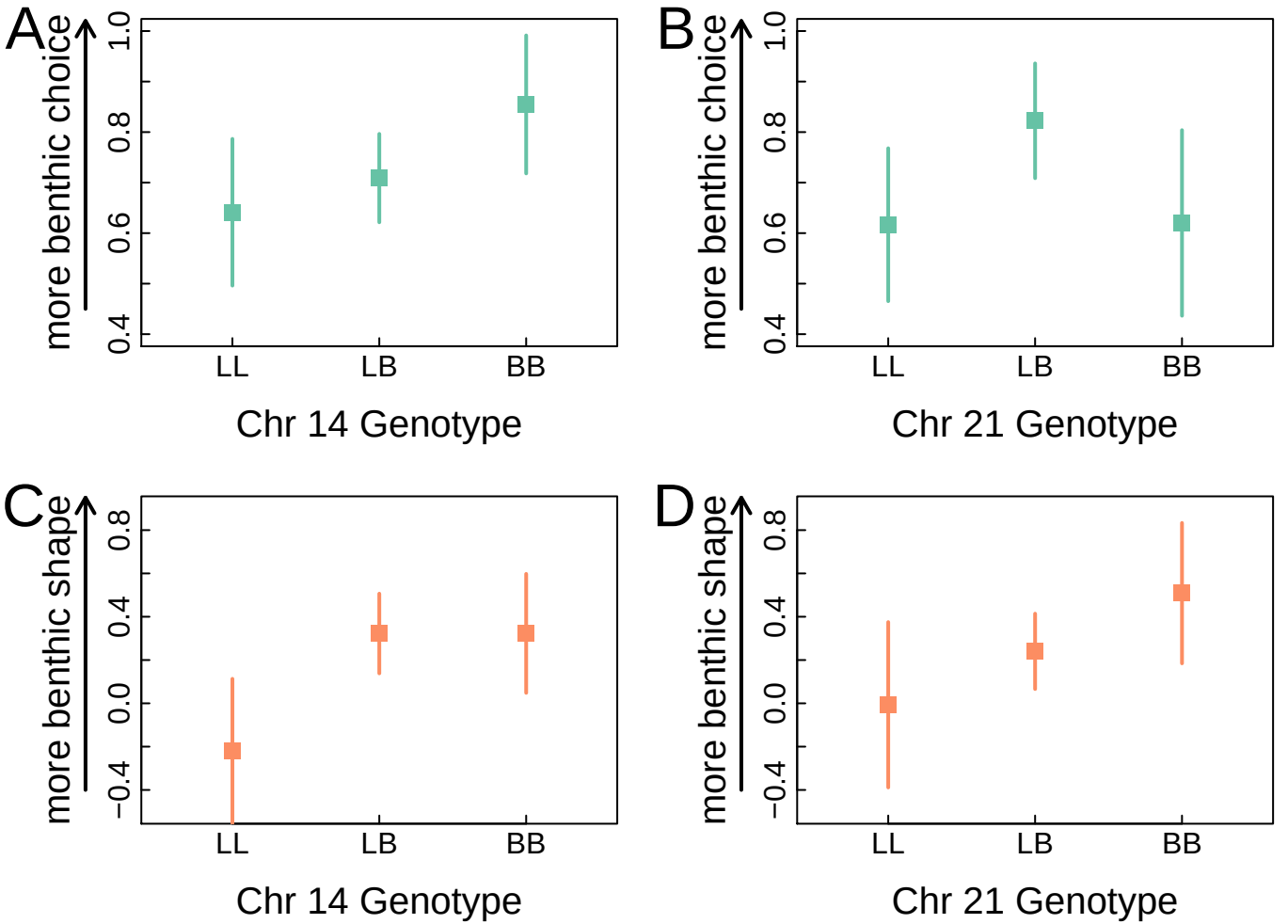
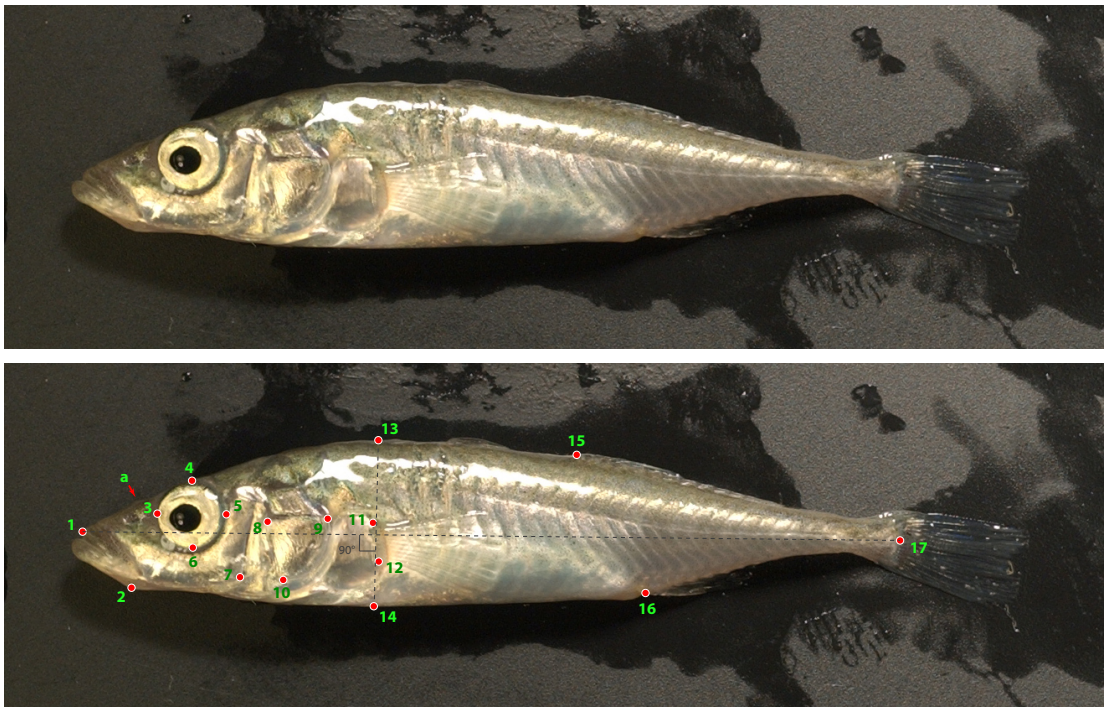

A
mate choice
LOD
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome
B
morphology PC2
LOD
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome
C
centroid size
LOD
0
1
2
3
4
5
6
7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Chromosome

Figure 3

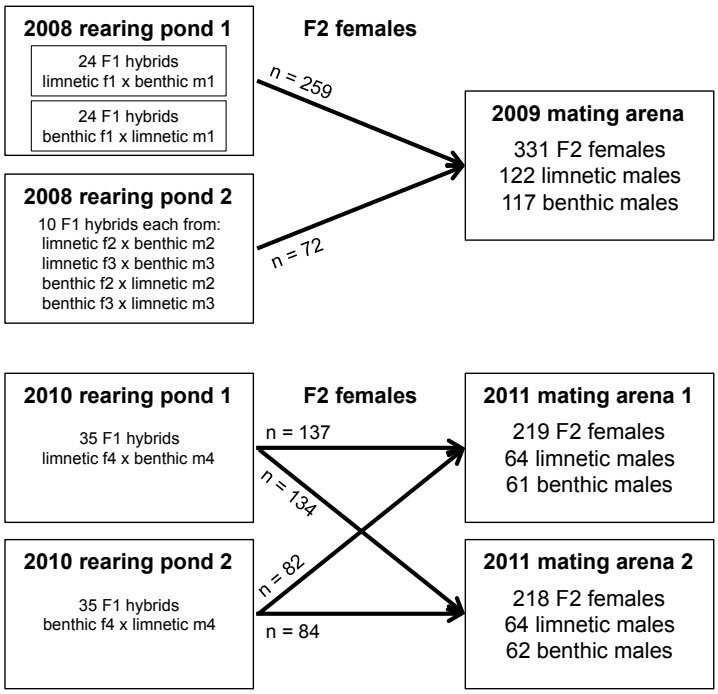

A
more benthic choice
1.0
0.8
0.6
0.4
LL
LB
BB
Chr 14 Genotype
B
more benthic choice
1.0
0.8
0.6
0.4
LL
LB
BB
Chr 21 Genotype
C
more benthic shape
0.8
0.4
0.0
−0.4
LL
LB
BB
Chr 14 Genotype
D
more benthic shape
0.8
0.4
0.0
−0.4
LL
LB
BB
Chr 21 Genotype



1. anterior tip of the upper jaw (anterior-most extent of the premaxilla; see a, below)
2. posterior-ventral corner of the lower jaw (border between the angular-articular and quadrate bones)
3. anterior-most point (edge) of the orbit
4. dorsal-most point (edge) of the orbit
5. posterior-most point (edge) of the orbit
6. ventral-most point (edge) of the orbit
7. inner corner of the preoperculum
8. anterior-dorsal corner of the operculum
9. posterior-dorsal corner of the operculum
10. ventral corner of the operculum
11. dorsal insertion of the pectoral fin
12. ventral insertion of the pectoral fin
13. dorsum of the trunk over the pectoral fin midpoint
14. ventrum of the trunk under the pectoral fin midpoint
15. anterior insertion of the dorsal fin
16. anterior insertion of the anal fin
17. posterior midpoint of the caudal peduncle

a. posterior end of the premaxilla's ascending process (the premaxilla is V-shaped)

Figure S1. Locations of 17 landmarks used in morphometric analysis. Related to Figure 1.



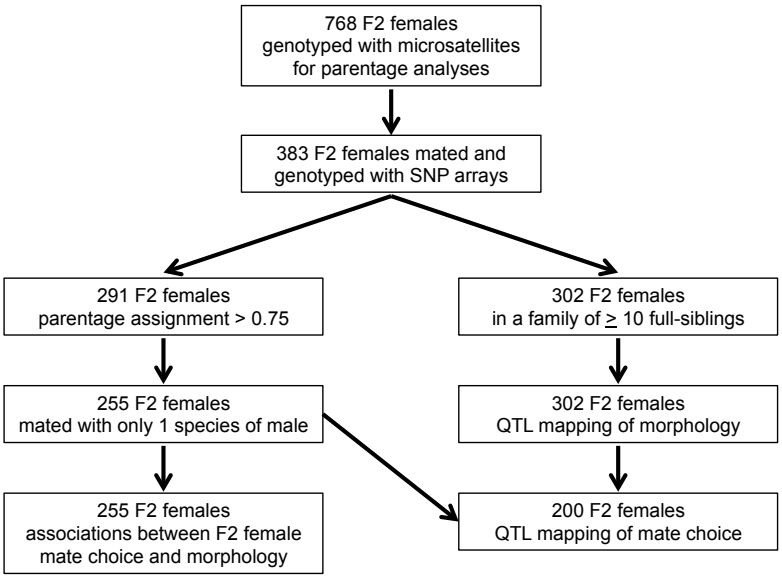


Figure S2. Overview of experimental design and analysis. Related to STAR Methods.

Landmark	PC2	LD1
x1	0.216	26.839
y1	-0.317*	4577.351*
x2	0.221	-744.582
y2	-0.272*	3823.246*
x3	0.030	221.469
y3	0.111	3577.608*
x4	-0.083	722.195
y4	0.023	3117.165*
x5	-0.166	242.918
y5	0.137	2624.617
x6	-0.061	-231.759
y6	0.208	3100.080
x7	-0.004	-589.378
y7	-0.054	2447.137
x8	-0.125	211.181
y8	0.184	2010.066
x9	-0.060	220.541
y9	0.149	1341.069
x10	-0.020	-571.613
y10	0.040	1866.242
x11	0.071	92.302
y11	0.076	659.106
x12	0.019	-420.228
y12	0.025	514.774
x13	0.048	1215.315
y13	0.229	550.743
x14	0.042	-1044.044
y14	-0.369*	579.358
x15	-0.203	1069.442
y15	0.277*	-1922.137
x16	0.256	-726.822
y16	-0.314*	-2864.858
x17	-0.179	181.686
y17	-0.133	-6244.713*

Table S1. Scaling of principal component axis 2 (PC2) and linear discriminant function 1 (LD1) by morphological landmarks. Related to Figure 1. Note that for visualization purposes, LD1 scores are multiplied by -1 so that directionality corresponds with PC2; a more benthic phenotype is indicated by higher and positive numbers. For each analysis, the top five landmarks are indicated with an asterisk.

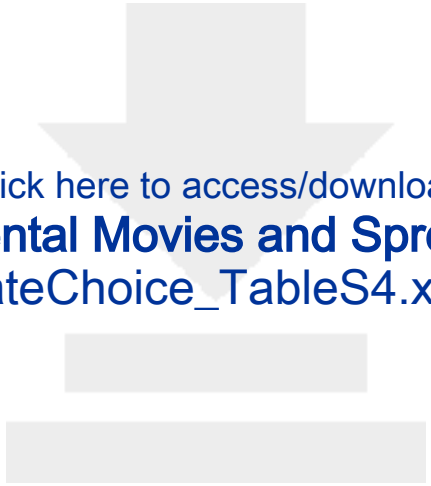
Trait	n	LOD	Chr	Position (cM)	Nearest SNP	Mean ± SE (LL)	Mean ± SE (LB)	Mean ± SE (BB)
mate choice	200	4.5	14	22.43	chrXIV:1713227	0.641 ± 0.08	0.706 ± 0.04	0.863 ± 0.06
mate choice	200	4.61	21	8	chrXXI:9373717	0.58 ± 0.06	0.83 ± 0.05	0.614 ± 0.11
discriminant function	302	4.83	12	17	chrXII:7504339	-0.15 ± 0.13	0.249 ± 0.08	0.54 ± 0.13
discriminant function	302	4.23	14	8.1	chrXIV:4632223	-0.228 ± 0.17	0.279 ± 0.08	0.393 ± 0.12
PC2	302	4.04	4	30.76	chrIV:11367975	-0.012 ± 0.008	0.012 ± 0.004	0.019 ± 0.006
PC2	302	6.67	7	47	chrVII:26448674	0.02 ± 0.007	0.015 ± 0.005	-0.013 ± 0.008
centroid size	302	6.97	9	47.8	chrIX:19745222	4.868 ± 0.12	5.074 ± 0.04	5.133 ± 0.08
x2*	302	3.93	7	60	chrUn:29400087	-1.208 ± 0.004	-1.21 ± 0.002	-1.198 ± 0.004
y2*	302	9.99	4	32	chrIV:11367975	-0.309 ± 0.003	-0.328 ± 0.002	-0.334 ± 0.003
x3	302	4.45	1	32.3	chrI:15145305	-1.101 ± 0.002	-1.094 ± 0.001	-1.089 ± 0.002
x4	302	5.13	16	30.9	chrXVI:12111717	-0.881 ± 0.002	-0.889 ± 0.001	-0.891 ± 0.002
x5*	302	4.54	15	6	chrXV:505537	-0.666 ± 0.003	-0.675 ± 0.002	-0.669 ± 0.003
y5	302	4.21	4	24.9	chrIV:15721538	0.099 ± 0.002	0.101 ± 0.001	0.108 ± 0.001
x6	302	3.96	16	29.5	chrXVI:13588796	-0.877 ± 0.002	-0.885 ± 0.002	-0.885 ± 0.003
y6*	302	4.14	9	30.2	chrIX:18942598	-0.111 ± 0.003	-0.103 ± 0.002	-0.105 ± 0.003
y15*	302	5.3	2	27	chrII:19324477	0.499 ± 0.005	0.5 ± 0.004	0.479 ± 0.005
x16	302	5.49	7	60	chrUn:29400087	1.906 ± 0.006	1.883 ± 0.004	1.854 ± 0.007
x17	302	4.92	1	32.8	chrI:14261764	3.369 ± 0.004	3.38 ± 0.003	3.392 ± 0.004

Table S2. Significant QTL loci for mate choice and morphology. Related to Figures 2 and 3. For each QTL, the table shows the number of F2 females used in analysis (n), log odds ratio (LOD), chromosome (Chr), position in centiMorgans (cM), nearest SNP, and mean and standard errors (SE) for the trait estimated in each genotype category – limnetic homozygote (LL), heterozygote (LB), and benthic homozygote (BB). QTL significance (α=0.1) was determined based on 10,000 permutations. The landmark coordinates significantly associated with mate choice are highlighted with an asterisk.

Marker	Genotyped	Chr	Forward primer (5' to 3')	Reverse primer (5' to 3')
LG1_7.59	2009, 2011	1	TGGACGAGTGCCAACATAAA	TTTTGGCAGCTCGGAATATC
LG1_27.1	2009	1	GAAGGAGGTTGGACATAAAGG	CTGCCTGCTTCTCAAAATACC
Stn27	2009, 2011	2	TCCTCTTGGGACAGTTGAGC	CTGAGAAGCTGCAGGAAGCC
Stn20	2009, 2011	2	CCAGATCATGTGTAAACGGC	AAGGCTCAGCTGTGATCTGG
Stn32	2009, 2011	3	CAGATTTCTCTCCCAGACGG	TGTATGCGCAGTGAGTAGGG
Stn45	2009, 2011	4	ACGAGGGTTTGAGTCTCTCC	GTTGTTCAATCCATCCGTCC
Stn309	2009, 2011	4	AACTGTGCAGATCTATGCCG	GGAAGTTGTAAAGAAAGGCCG
Stn241	2009, 2011	5	GACCTCCAGAACCAGGAAGG	CTTTACCAAGGTGAGGGACG
Stn85	2009, 2011	8	ACAGGACACCAGTGTAGCCC	ATGAGCGTGTCTCTCTTCCC
Stn98	2009	8	CAAAGTGCACACTACGTCGC	AGTGGAATAAAGGGAACCCG
Stn225	2009, 2011	9	AACATCGGAGACCACTGACG	ACGAGGCAACTTCCTTCTGC
Stn119	2009, 2011	10	CTCTACTGCTTTCCTCCATGC	TGAGCCTTCACAGACCACC
LG11_4.0	2009, 2011	11	GGCCCATTAGAGTCATCAAGC	GCACATGAGTGAGAGTGTGC
Gac7033	2009, 2011	11	AGGTGGATTGGTTTTCTG	GGACGCTCGCTCTTTC
Stn148	2011	13	AACCCTTACTCAACTCAGCCC	GAGGAACTTCATTTGGCAGC
Stn163	2009, 2011	14	GAGAAGACAACAGGGAAGCG	CGCCTGCAGTCAACCTACC
LG15_13.4	2011	15	CAGGGTTTCACACTTCAACC	CACAGAATGGCTGATTACGC
Stn344	2009, 2011	17	TTTGTTGGGATCTGGAGACG	GAGCTCTTCAAGCTGGTTCC
Stn305	2011	18	TGATCCAACGGTCAGATTCC	GTTCACCTGGCGAGGACG
Stn290	2009, 2011	19	CATCCAGAGCCTGTTTGAGG	TCACGGACTGTGGATCAGC
Stn194	2009, 2011	19	ACACTCTGCTCTCGCTCCG	TGGAAAGGCTTACTGTTCCG

Table S3. Microsatellite markers used for parentage assignments. Related to Figures 1 and 2. For each marker, the mate choice experiment year in which that marker was genotyped, the chromosome (Chr), and the primer sequences are given.

Table S4



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Supplemental Movies and Spreadsheets

MateChoice_TableS4.xlsx