1 **Genetic coupling of female mate choice with polygenic ecological divergence**

2 **facilitates stickleback speciation**

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24 **SUMMARY**

25 Ecological speciation with gene flow is widespread in nature [1], but presents a

26 conundrum: how are associations between traits under divergent natural selection and

27 traits that contribute to assortative mating maintained? Theoretical models suggest that

28 genetic mechanisms inhibiting free recombination between loci underlying these two types

29 of traits (hereafter, "genetic coupling") can facilitate speciation [2-4]. Here, we perform a

30 direct test for genetic coupling by mapping both divergent traits and female mate choice in

31 a classic model of ecological speciation: sympatric benthic and limnetic threespine

32 stickleback (*Gasterosteus aculeatus*). By measuring mate choice in F2 hybrid females, we

33 allowed for recombination between loci underlying assortative mating and those under

34 divergent ecological selection. In semi-natural mating arenas in which females had access

35 to both benthic and limnetic males, we found that F2 females mated with males similar to

36 themselves in body size and shape. In addition, we found two quantitative trait loci (QTL)

37 associated with female mate choice that also predicted female morphology along the

38 benthic-limnetic trait axis. Furthermore, a polygenic genetic model that explains adaptation

39 to contrasting benthic and limnetic feeding niches [5] also predicted F2 female mate choice.

40 Together, these results provide empirical evidence that genetic coupling of assortative

41 mating with traits under divergent ecological selection helps maintain species in the face of

42 gene flow, despite a polygenic basis for adaptation to divergent environments.

43

44 **RESULTS**

45 We tested for genetic coupling between loci underlying ecologically divergent traits and

46 assortative mating by examining morphological and genomic determinants of female mate

47   choice in a sympatric pair of benthic and limnetic threespine stickleback from Paxton Lake

48   in British Columbia, Canada. Species pairs of stickleback have evolved repeatedly in

49   multiple postglacial lakes in British Columbia [6,7]. Each lake contains a larger, deeper

50   bodied benthic form that inhabits inshore habitats, and a smaller, shallow bodied limnetic

51   form that inhabits open water [8,9]. These species are morphologically adapted to their

52   contrasting food sources: benthic stickleback primarily feed on invertebrates inhabiting the

53   substrate or attached to vegetation, whereas limnetics specialize on zooplankton [10-12].

54   Although hybrids exist in the wild [13-15] and there are no strong intrinsic

55   incompatibilities [14,16], benthics and limnetics show nearly complete assortative mating

56   in experimental trials [17]. Previous no-choice mating trials suggested that benthic and

57   limnetic females prefer mates with similar body size [18-20] and shape [20]. In this study,

58   we conducted female mate choice experiments in ponds that allowed females to access

59   both benthic and limnetic males in habitats that closely mimic those found in the wild [5].

60   By examining whether recombinant F2 hybrid females that vary in phenotype mate with

61   benthic or limnetic males, we tested whether females prefer to mate with individuals that

62   have similar phenotypes to themselves. We also identified QTL for female mate choice and

63   morphology to test whether genomic regions associated with mate choice correspond to

64   regions determining phenotypic traits under divergent selection. These represent the first

65   direct tests of genetic coupling in this vertebrate system.

66

67   **F2 females prefer males with a similar body shape and size**

68   Body shape of F2 hybrid females was positively associated with the shape of chosen mates.

69   We defined shape based on 17 external morphological landmarks (34 x- and y-coordinates;

3

70   Figure S1), with landmarks for each fish rotated and scaled to the same centroid size. We

71   used principal component (PC) analysis of the morphological landmark coordinates to

72   summarize continuous variation in phenotypes of pure-species males and F2 females,

73   which allowed us to examine shape variation associated with female choice within and

74   between benthic and limnetic males. The first PC axis (PC1) separated male from female

75   fish and was not analyzed further. PC2 separated benthic males from limnetic males, with

76   F2 females intermediate (Figure 1A). Females that mated with limnetic males had lower

77   (more limnetic-like) PC2 shape values than those that mated with benthic males (χ^2=17.46;

78   P=2.9×10^{-5}: partial R^2=0.072; Figure 1B). Remarkably, among F2 females that mated with

79   benthic males, those most benthic-like in shape tended to mate with benthic males that

80   were closer to the benthic extreme of the PC2 shape distribution ($F_{1,444}$=6.65; P=0.01;

81   partial R^2=0.015; Figure 1C). We did not detect a similar trend in F2 females that mated

82   with limnetic males ($F_{1,444}$=0.33; P=0.56; partial R^2=0.0008; Figure 1C). We also analyzed

83   F2 female shape using a discriminant function that separates benthic from limnetic males

84   based on the 34 external morphological landmark coordinates (Table S1). In accordance

85   with the results above using PC2, females that mated with benthic males had a more

86   benthic-like shape than those that mated with limnetic males (χ^2=16.23; P=5.6×10^{-5}; partial

87   R^2=0.065). When centroid size was used as a covariate in these analyses, the correlations

88   between the shape of F2 hybrid females and the chosen males remained (data not shown),

89   suggesting that body shape is an important component of female mate choice.

90

91   Body size of hybrid F2 females also predicted mate choice. Females with larger centroid

92   sizes preferentially mated with males of the larger, benthic species (χ^2=17.79; P=2.5×10^{-5};

4

93   partial R^2=0.103; Figure 1D). Among F2 females that mated with benthic males, there was a

94   non-significant tendency for the largest of them to mate with the largest benthic males

95   ($F_{1,444}$=1.89; P=0.17; partial R^2=0.004; Figure 1E). There was a similar positive tendency

96   among F2 females that mated with limnetic males, though again this pattern was not

97   significant ($F_{1,444}$=1.06; P=0.30; partial R^2=0.002; Figure 1E).

98

99   Eight of 34 shape traits (x- and y-coordinates of 17 morphological landmarks; Figure S1),

100   found mainly in the head and the caudal region of F2 females, were significantly associated

101   with mate choice when tested one at a time: y1, x2, y2, x5, y6, y9, y15, and y16 (FDR-

102   adjusted P<0.05). The importance of some of these traits to mate choice is also indicated by

103   their contribution to scaling on the benthic-limnetic discriminant function and their

104   loading on PC2 (Table S1). The scaling values of two jaw coordinates (y1 and y2) are within

105   the top five scaling values on the first linear discriminant axis. Both of those coordinates,

106   along with coordinates at the insertion of the dorsal (y15) and anal (y16) fins, were also

107   within the top five loadings on PC2.

108

109   **Genetic coupling of mate choice and ecological traits**

110   We found two QTL peaks for F2 female mate choice (Figure 2A; Table S2), on chromosomes

111   14 (LOD= 4.5, PVE=7.52) and 21 (LOD=4.61, PVE=10.07). For the QTL on chromosome 14,

112   F2 females homozygous for the benthic allele (BB) were more likely to choose a benthic

113   mate than either the limnetic homozygotes (LL) or heterozygotes (LB) (Figure 3; means:

114   LL=0.64, LB=0.71, BB=0.86, where 0 and 1 indicate limnetic and benthic mate choice,

115   respectively). The QTL on chromosome 21 showed a different pattern, where the

116 heterozygote was more likely to choose a benthic mate than either homozygote (means:

117 LL=0.58, LB=0.83, BB=0.61).

118

119 F2 female mate choice is associated with her own shape and size, despite the opportunity

120 for recombination between loci underlying the traits, suggesting either pleiotropy or

121 physical linkage between morphology and mate choice loci. For this reason, we also

122 investigated the genetic architecture of F2 female morphology. The results suggest that the

123 genetic basis of morphological traits correlated with mate choice is more widely

124 distributed across the genome than implied by the two QTL we identified for mate choice.

125 Of the QTL for body size and the three measures of shape variation predicting mate choice

126 (i.e. eight x- and y-landmark coordinates, PC2, and the benthic-limnetic linear discriminant

127 function), a single QTL for body shape overlaps with a mate choice QTL (Table S2). We

128 found a single QTL on chromosome 9 for centroid size (Figure 2C; LOD=6.97, PVE=10.08)

129 and two QTL for PC2: one on chromosome 4 (LOD=4.04; PVE=5.97) and one on

130 chromosome 7 (LOD=6.67; PVE=9.71). Of the eight landmark traits correlated with mate

131 choice, five were influenced by QTL distributed across five chromosomes (Table S2). One of

132 these QTL, for a jaw landmark coordinate (y2), overlapped with the QTL for PC2 on

133 chromosome 4. Finally, two QTL were associated with the discriminant function separating

134 benthic and limnetic morphology (Figure 2B). One of the QTL overlapped with the mate

135 choice QTL on chromosome 14 (LOD=4.23; PVE=6.24), and the other mapped to

136 chromosome 12 (LOD=4.83; PVE=7.10). At both QTL, the benthic allele was associated with

137 a higher (more benthic-like) value of the morphological trait (Table S2).

138

139   Despite this distributed genetic architecture for F2 female body size and shape, two lines of

140   evidence suggest genetic coupling between the QTL detected for mate choice and those

141   detected for ecologically divergent traits. First, a linear model containing the two QTL

142   detected for mate choice on chromosomes 14 and 21 explained a significant amount of

143   variation in the benthic-limnetic discriminant function (Figure 3; P=0.015; LOD=2.73;

144   PVE=4.08). Second, an additive, polygenic QTL model that predicted F2 hybrid position

145   along the benthic-limnetic ecological niche axis provided by an earlier study of the same

146   species pair [5] also accounted for a significant proportion of the variance in mate choice in

147   the current study (P=0.001; LOD=10.48; PVE=21.4). The linear model based on these QTL

148   genotypes also explained a significant proportion of the variance in the benthic-limnetic

149   discriminant function in our experiment (P=0.00005; LOD=13.24; PVE=18.29).

150

151   **DISCUSSION**

152   The genetic basis of mate choice has consequences for the efficacy of ecological speciation

153   with gene flow. We used data on associations between morphology, genetics, and mate

154   choice to test predictions of the "genetic coupling" model for the evolution of mate choice.

155   We investigated the genetic basis of interspecific mate choice in a sympatric species pair of

156   stickleback that continue to undergo a low level of hybridization in the wild [13-15]. By

157   measuring mate choice in F2 hybrids, which allowed the opportunity for some

158   recombination between loci encoding mate choice and those encoding traits under

159   divergent selection, we found strong evidence for genetic coupling. First, we found that F2

160   hybrid females mated with males that were more similar to themselves in shape and size.

161   This result implies that assortative mating between like phenotypes was not eliminated by

162   recombination in this hybrid population. Second, we found two QTL for mate choice that

163   also explained variation in body shape. Finally, we found that a QTL model that explained

164   variation in F2 hybrid niche use along the benthic-limnetic axis in a previous study [5] also

165   explained variation in both F2 female shape and mate choice in our study. Together, these

166   results are consistent with genetic coupling for the evolution and maintenance of

167   assortative mating in this stickleback species pair.

168

169   The absence of free recombination between loci for mate choice and loci for traits under

170   divergent selection (i.e. genetic coupling) could be due to either pleiotropy or close linkage.

171   Felsenstein [2] showed that both mechanisms increase the likelihood of speciation and

172   species persistence in the face of gene flow. Pleiotropy can result from phenotype

173   matching, whereby individuals in both species (and their hybrids) prefer to mate with

174   individuals having a similar phenotype to their own. This corresponds to Felsenstein's

175   "one-allele" model for the evolution of mate choice, because at a given mating locus the

176   same allele encodes conspecific preference in both species (e.g., it encodes a phenotype

177   matching behavior "mate with like"). When the phenotype matching alleles are fixed in

178   both species, the observed genetic determinants of variation in mate choice are the allelic

179   variants at the loci underlying traits upon which matching is based. This contrasts with

180   Felsenstein's "two-allele" model with linkage, in which distinct alleles controlling

181   assortative mating between alternative phenotypes are physically linked to genes for traits

182   under divergent natural selection.

183

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,

207   which possess a much lower amount of size and shape variation than is seen between the

208   two parent species, thus reducing the opportunity for imprinting or learning. It is possible

209   to imagine that during courtship a female would be capable of evaluating her own body

210   size relative to that of a male, but it seems far less plausible that she would be able to

211   compare subtle differences in their body shapes. Instead, phenotype matching might occur

212   not by direct comparison of morphology but rather by a shared feeding habitat preference

213   between individuals that are similar in morphology. In threespine stickleback, size and

214   shape is strongly associated with niche use both among species and among F2 hybrid

215   individuals varying in morphology [5,10-12]. For example, the most benthic-like F2 females

216   might feed preferentially in the same pond regions as do male benthics, and this higher

217   encounter rate between like individuals might then lead to a higher probability of mating.

218

219   Regardless of the underlying mechanism, our results provide empirical evidence that

220   genetic coupling is important for the persistence of species in the face of gene flow.

221   Although genetic coupling, either via a one-allele mechanism [23] or a two-allele

222   mechanism with linkage, has now been shown in a few other systems, in all of these cases

223   the divergent traits are encoded by one or a few loci of relatively large effect [24-30].

224   However, such a simple genetic architecture for traits under divergent selection might be

225   relatively rare. Our previous studies in stickleback have indeed shown that the genetic

226   architecture of adaptation in this system is highly polygenic [5,31,32]. This diffuse genetic

227   architecture of adaptation makes a two-allele model with tight linkage seem less plausible,

228   because this would require a large number of mate choice alleles to be distributed across

229   the genome, all in tight linkage with alleles for traits under divergent selection. Under

230   either model, our results suggest that even when the underlying genetic architecture of

231   phenotypes under divergent selection is polygenic and distributed across the genome,

232   genetic coupling with assortative mating will contribute to the persistence of species in the

233   face of gene flow.

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AUTHOR CONTRIBUTIONS

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360 **Figure 1. Mate choice of F2 females is associated with shape and centroid size of**

361 **males and females.** Shape is summarized using principal component analysis of 17

362 landmarks. (A) PC2 separates benthic and limnetic males, with F2 females intermediate. In

363 F2 females, PC2 is significantly associated with the male species chosen (B) and with

364 variation in male PC2 scores when benthic males were chosen (C). In F2 females, centroid

365 size is significantly associated with mate choice (D), but not with variation in male centroid

366 size when benthic males were chosen or when limnetic males were chosen (E). See also

367 Figure S1, Table S1, Table S3.

368

369 **Figure 2. QTL mapping of female mate choice, body shape, and body size.** The graphs

370 show LOD scores across the 21 stickleback chromosomes for: (A) female mate choice, (B)

371 benthic-limnetic discriminant function, and (C) centroid size. Dotted lines: $\alpha=0.1$ genome-

372 wide significance cutoff based on 10,000 permutations. See also Table S2, Table S3, Table

373 S4.

374

375 **Figure 3. Effects of two QTL on female mate choice and body shape.** The effects of the

376 mate choice QTL on chromosome 14 (A,C) and 21 (B,D) are shown for mate choice (A,B)

377 and shape, represented by discriminant function score (C, D). QTL for mate choice is based

378 on a binary response variable with 0=limnetic and 1=benthic. Points represent mean for

379 each female genotype and error bars indicate 95% confidence intervals. See also Table S2.

380

STAR METHODS

382

383 **CONTACT FOR REAGENT AND RESOURCE SHARING**

384 Further information and requests for resources and reagents should be directed to and will

385 be fulfilled by the Lead Contact, Catherine Peichel (catherine.peichel@iee.unibe.ch).

386

387 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

388 All animal experiments involved threespine stickleback (*Gasterosteus aculeatus*) fish and

389 were approved by the University of British Columbia Animal Care Committee (protocols

390 A07-0293, A11-0402) and the Fred Hutchinson Cancer Research Center Institutional

391 Animal Care and Use Committee (protocol 1797).

392

393 **Generation of F2 females**

394 In 2007, we used wild-caught adult fish to make six *in vitro* interspecific crosses. Three

395 crosses involved a limnetic female and three crosses involved a benthic female. We stored

396 their bodies in 95% ethanol for DNA analysis. We reared the resulting F1 hybrids in the

397 laboratory. In March 2008, F1 hybrids were introduced to two outdoor experimental ponds

398 on the campus of University of British Columbia (described in [5]). For the first, we

399 randomly selected 24 F1 hybrid adults from a cross involving a limnetic female, and 24 F1

400 hybrid adults from a cross involving a benthic female. We took a sample of caudal fin tissue

401 from each individual F1 hybrid for DNA analysis and then released them into two separate

402 mesh enclosures within a pond. The enclosures were designed to allow only full-sib

403 matings between F1s and to allow F2 hybrid offspring to escape the enclosure into the

404   pond. However, we realized that the enclosures were limiting the number of F2 hybrids

405   that were produced. Thus, we established a second rearing pond, for which we randomly

406   selected five F1 hybrids of each sex from the remaining four crosses. We took a sample of

407   caudal fin tissue from each individual for DNA analysis and then released them into the

408   pond. This design allowed interbreeding between F1s from different crosses. In 2009, we

409   used wild-caught adult fish to make two additional *in vitro* interspecific crosses. One cross

410   involved a limnetic female and the other a benthic female. We stored their bodies in 95%

411   ethanol for DNA analysis. We reared the resulting F1 hybrids in the laboratory. In May

412   2010, we initiated two F2 rearing ponds to increase the number of F2 hybrids generated

413   and to allow only full-sib matings between F1 hybrids. We randomly selected 35 F1 hybrid

414   adults from the cross involving a limnetic female and 35 F1 hybrid adults from the cross

415   involving a benthic female. We took a sample of caudal fin tissue from each individual F1

416   hybrid for DNA analysis and then released them into their respective ponds. After release,

417   the F1 hybrids were allowed to mate freely with their full-siblings in the same pond

418   throughout the breeding season. For an overview of the source and numbers of the F2

419   females used in these experiments in both years, see Figure S2.

420

421   The ponds (25 x 15 m surface area) contained a sloping shallow zone and a deep open-

422   water zone (6 m deep), thereby providing feeding and nesting habitat for both species [5].

423   In each spring of 2007 – 2010, we inoculated the ponds with macrophytes, sediments and

424   water full of aquatic insects, mollusks and plankton from Paxton Lake. Each time we added

425   1.25kg of a 25.5:1 mix of 50% pure KNO_3 : KH_2PO_4 to stimulate primary production.

426

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%

450   ethanol. When multiple clutches were found within the same nest (determined visually via

451   different egg clumps and extent of egg development), each clutch was treated separately.

452

453   **Parentage assignment**

454   For the mate choice experiment conducted in 2009, we genotyped 331 F2 females, 117

455   benthic males, 122 limnetic males, and 245 fertilized eggs (1 per clutch) or free-swimming

456   juveniles with 18 microsatellite markers (Table S3) following [33]. For the mate choice

457   experiment conducted in 2011, we genotyped 437 F2 females (219 in arena 1, 218 in arena

458   2), 123 benthic males (61 in arena 1, 62 in arena 2), 128 limnetic males (64 in arena 1, 64

459   in arena 2), and 328 fertilized eggs (1 per clutch) or free-swimming juveniles (186 in arena

460   1, 142 in arena 2) with 19 microsatellite markers (Table S3). Parentage was assigned using

461   the R package 'MasterBayes' [34] with the following parameters: E1=0.01, E2=0.01,

462   mm.tol=10 (DRYAD data file 'pedigree.all.csv').

463

464   In total, 383 unique F2 females were identified in the parentage analyses (Figure S2).

465   However, for further analyses, we only considered the 467 unique mating events for which

466   the probability of parentage assignment of a fertilized egg or free-swimming juvenile was

467   greater than 0.75. Using these assignments, we assessed mate choice for 291 unique F2

468   females, of which 255 mated exclusively with a single male species while the remaining 36

469   chose males of the two species for separate clutches (DRYAD data file 'choice.all.csv'). Of

470   the 255 F2 females that mated with only one species, 191 mated once, while 64 mated

471   multiple times including one F2 female that mated with benthic males ten times.

472

473　**Morphological analysis**

474　We used 17 morphological landmarks to summarize morphology in wild-caught benthic

475　and limnetic males and F2 females (Figure S1; DRYAD data file 'phenotypes.all.csv'). Using

476　digital images taken of live fish alongside a ruler for scale, we recorded the x- and y-

477　coordinates of each landmark and scaled the values using 'tpsDig' v2.12 [35]. Coordinates

478　were superimposed, and scaled values as well as centroid sizes were calculated using

479　Generalized Procrustes Analysis in the R package 'shapes' [36]. We summarized these

480　landmarks using principal component analysis with the 'prcomp' function in R [37].

481　Custom R scripts ('Morphology.R' and 'landmarks.R') for these analyses are provided on

482　DRYAD (http://dx.doi.org/10.5061/dryad.bs7sg).

483

484　**Association of F2 mate choice and morphology**

485　We tested for associations between mate choice and morphology of the 255 F2 females that

486　mated with only a single male species using centroid size as a measure of body size and

487　three measures of F2 female shape based on landmarks: (1) principal component analysis,

488　(2) discriminant function analysis; (3) individual x and y coordinates of landmarks. For

489　centroid size, we tested associations between F2 female size and female mate choice using

490　a binomial generalized linear model with experimental pond as a covariate.

491

492　*Principal component analysis*

493　We used principal component analysis to examine morphological variation within F2

494　females as well as within and between benthic and limnetic males. A single principal

495　component axis (PC2) separated benthic and limnetic males, with F2 females intermediate.

496   We used this benthic-limnetic PC axis to test associations between female morphology and

497   female mate choice. Parents of each egg clutch, as determined from the parentage analysis,

498   were used to determine the species of male chosen by each F2 female. We used a binomial

499   generalized linear model to test associations between female mate choice (benthic or

500   limnetic) and her score along the benthic-limnetic PC axis, with experimental pond as a

501   covariate and significance assessed using the drop1 function in R. We also used linear

502   models to compare female PC scores with the PC scores of the chosen males, with

503   experimental pond as a covariate and mother as a random effect. Coefficients of partial

504   determination (partial R^2) were calculated using the 'rsq' package in R [37]. We repeated

505   these analyses using centroid size as a measure of body size in place of the benthic-limnetic

506   PC axis.

507

508   ***Discriminant function analysis***

509   We used discriminant function analysis to summarize F2 female shape morphology along a

510   benthic-limnetic axis. We used morphological landmarks from wild-caught benthic and

511   limnetic males to build a discriminant function with the R package 'MASS' [38]. This model

512   had 99.8% classification accuracy using 10-fold cross-validation; only a single individual

513   male was incorrectly classified. The model was used to predict discriminant function values

514   for F2 females based on the same morphological landmarks. We then tested for association

515   between this benthic-limnetic discriminant function value and mate choice in F2 females

516   using a binomial generalized linear model with experimental pond as a covariate.

517

518   ***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

542 × F1 family having at least 10 full-siblings for linkage map construction and subsequent

543 QTL analyses, resulting in the inclusion of 302 F2 females from this experiment. We first

544 calculated pairwise recombination frequencies for each F1 × F1 family using JoinMap ver

545 3.0 [40]; recombination frequencies were concatenated and imported into JoinMap to

546 produce a single linkage map. We found 21 linkage groups, which were assigned to the 21

547 chromosomes from the stickleback genome assembly using known SNP locations.

548

549 **QTL analysis**

550 All QTL analysis was performed in the 'R/qtl' package [41], and a custom R script 'QTL.R' is

551 provided on DRYAD (http://dx.doi.org/10.5061/dryad.bs7sg). Although power to detect

552 QTL of small effect is increased by having more individuals, power to detect QTL at all is

553 reduced if the phenotypic analysis is not robust. To map mate choice, we therefore

554 conservatively used the 200 F2 females that were: (1) included in the linkage map

555 construction; (2) had a parentage assignment probability greater than 0.75; and (3) mated

556 with only a single species of male (DRYAD data files: 'purechoice.gen.csv' and

557 'purechoice.pheno.csv'). We used the 'scanone' command with Haley-Knott regression and

558 a binary response variable (1 = chose benthic; 0 = chose limnetic), with both family and

559 experimental pond as covariates. To determine significance, we used 10,000 permutations

560 and a genome-wide cutoff of $\alpha=0.1$. We used this lenient threshold because our main goal

561 was to determine whether QTL for mate choice and morphology lie in the same regions, so

562 false positives were less of a concern than missing QTL.

563

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]

586   to explain the predicted benthic-limnetic discriminant function value as well as mate choice

587   in the 200 F2 females used to map mate choice.

588

589   **QUANTIFICATION AND STATISTICAL ANALYSIS**

590   All analysis was conducted in R [37]. Statistical tests and software used are described in

591   Method Details (above).

592

593   **DATA AND SOFTWARE AVAILABILITY**

594   All data files and custom R scripts required to recreate these analyses are available on

595   DRYAD: http://dx.doi.org/10.5061/dryad.bs7sg.

596

597   **SUPPLEMENTAL INFORMATION**

598   Supplemental Information PDF contains 2 figures and 3 tables.

599

600   **Table S4. Names and locations of SNPs used for linkage mapping and QTL analysis.**

601   **Related to Figure 2.** The positions in bp refer to the original threespine stickleback

602   genome assembly (Broad S1, Feb. 2006;

603   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

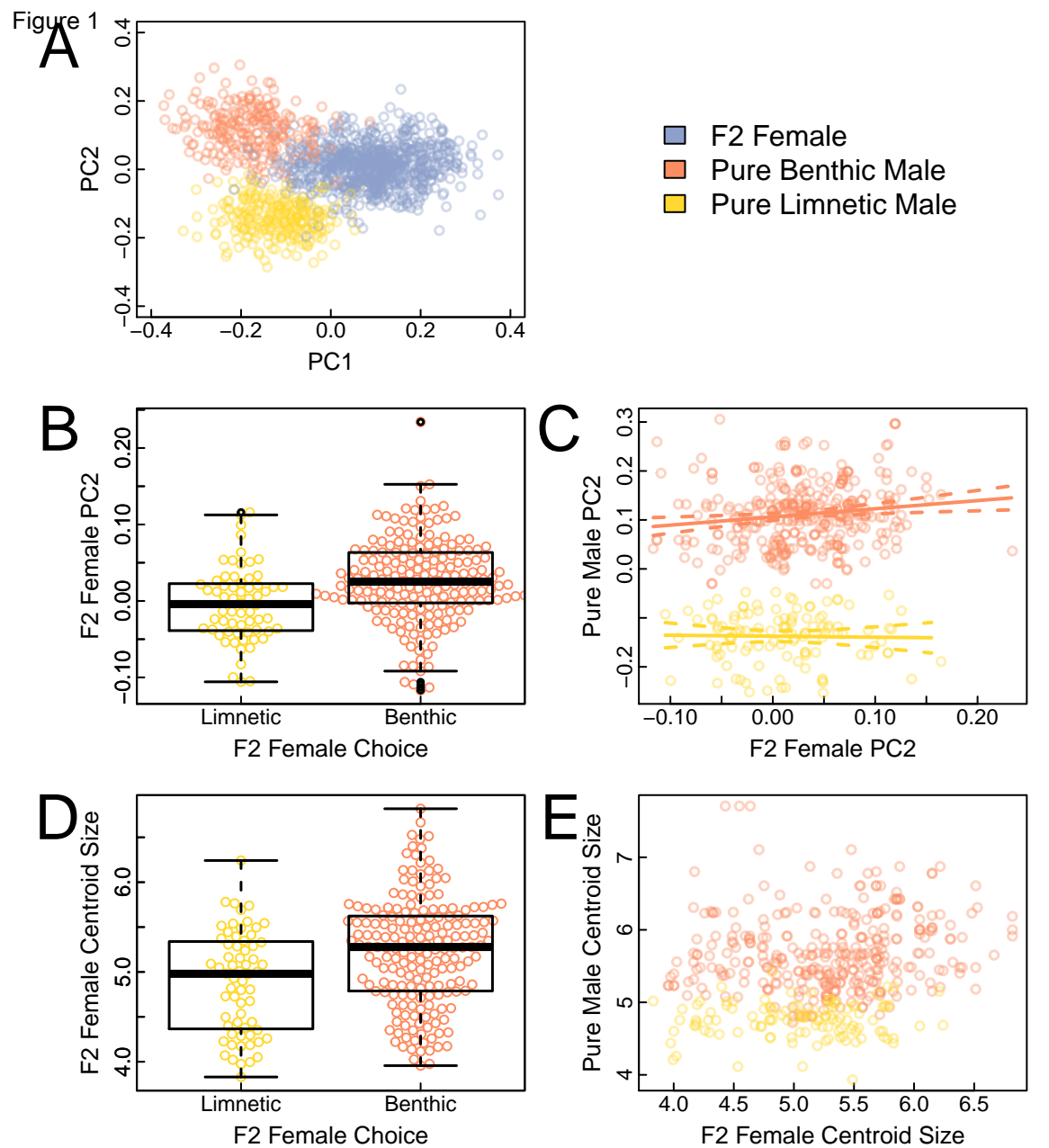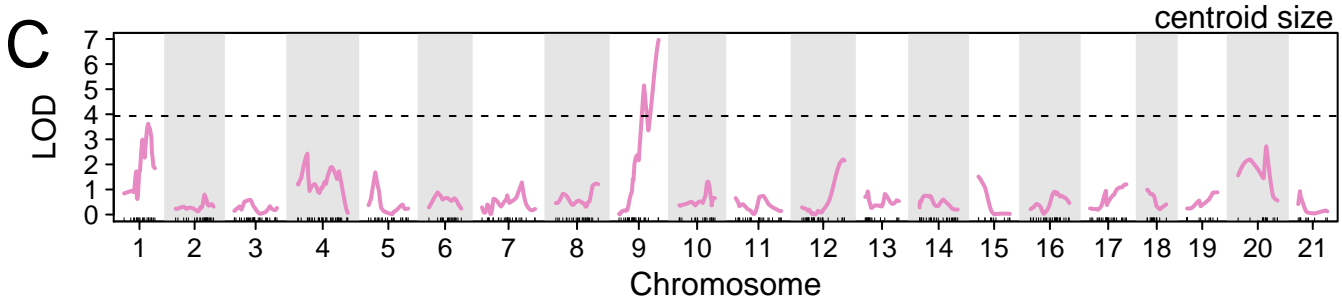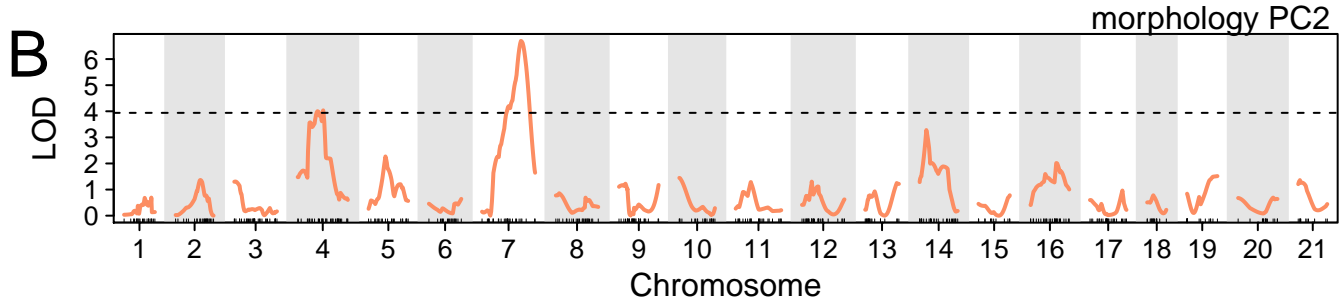|		
R package 'shapes'	[36]	https://www.maths.nottingham.ac.uk/personal/ild/shapes/
R core team	[37]	https://www.r-project.org/foundation/
R package 'MASS'	[38]	https://cran.r-project.org/web/packages/MASS/MASS.pdf
GenomeStudio	Illumina	https://support.illumina.com/array/array_software/genomestudio/downloads.html
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

Figure 2

Figure 3

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
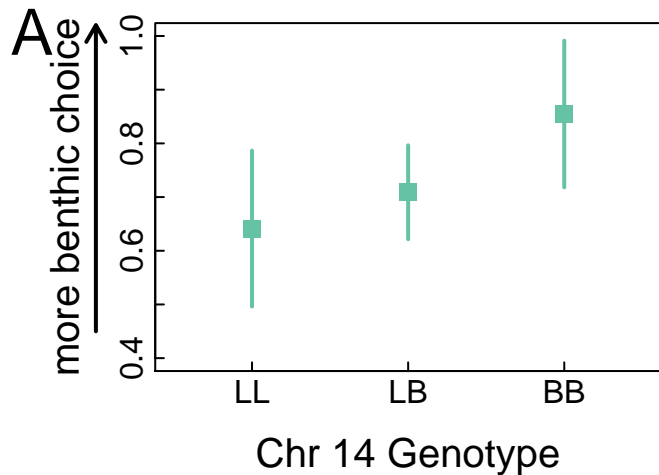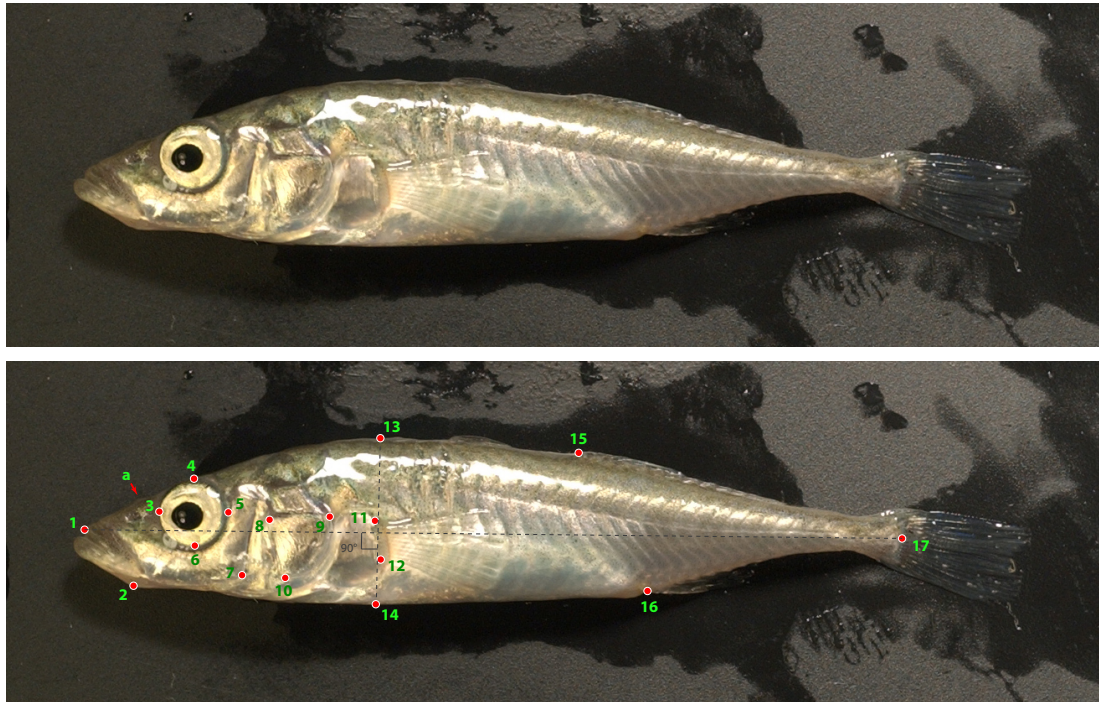7. inner corner of the preoperculum
8. anterior-dorsal corner of the operculum
9. posterior-dorsal corner of the operculum
10. ventral corner of the operculum
11. dorsal insertion of the pectoral fin
12. ventral insertion of the pectoral fin
13. dorsum of the trunk over the pectoral fin midpoint
14. 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.
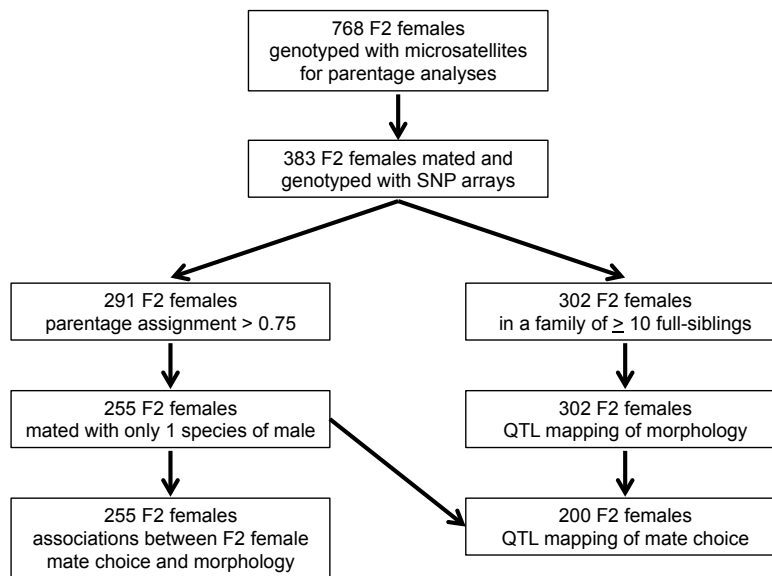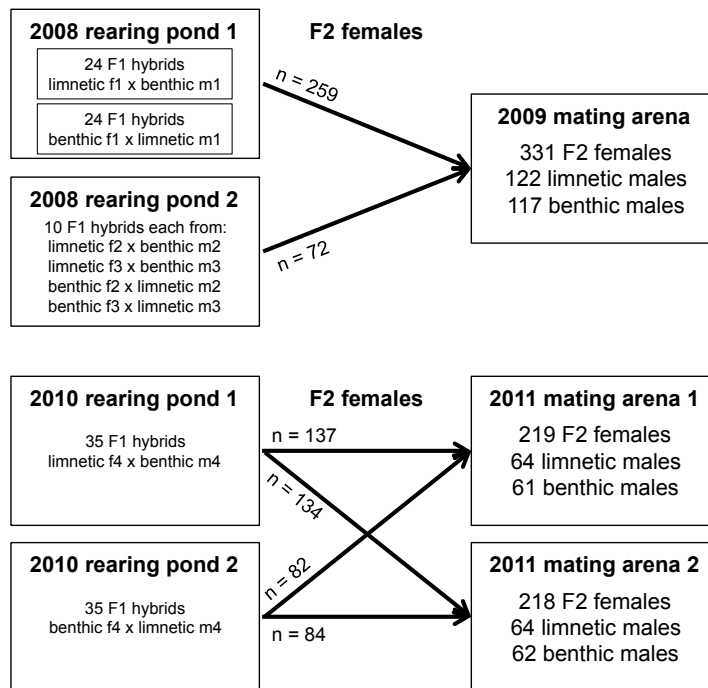
Figure S2. Overview of experimental design and analysis. Related to STAR Methods.

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