The genetic architecture of divergence between threespine stickleback species

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The genetic and molecular basis of morphological evolution is poorly understood, particularly in vertebrates. Genetic studies of the differences between naturally occurring vertebrate species have been limited by the expense and difficulty of raising large numbers of animals and the absence of molecular linkage maps for all but a handful of laboratory and domesticated animals. We have developed a genome-wide linkage map for the three-spined stickleback (*Gasterosteus aculeatus*), an extensively studied teleost fish that has undergone rapid divergence and speciation since the melting of glaciers 15,000 years ago[1]. Here we use this map to analyse the genetic basis of recently evolved changes in skeletal armour and feeding morphologies seen in the benthic and limnetic stickleback species from Priest Lake, British Columbia. Substantial alterations in spine length, armour plate number, and gill raker number are controlled by genetic factors that map to independent chromosome regions. Further study of these regions will help to define the number and type of genetic changes that underlie morphological diversification during vertebrate evolution.

Three-spined sticklebacks provide one of the best known examples of rapid adaptive radiation in vertebrates. A large number of distinct morphological forms of sticklebacks have evolved following the colonization of newly created coastal streams and lakes at the end of the last ice age[1]. In at least six lakes in coastal British Columbia, pairs of sympatric stickleback species have been identified. Members of a species pair are adapted to different niches within a lake, with corresponding changes in feeding morphology and defensive armour occurring in parallel in the different lakes (Fig. 1)[2]. The benthic species feeds on invertebrates near shore and has a great reduction in the amount of body armour, increased body depth, and a decreased number of gill rakers for filtering ingested food. The limnetic species more closely resembles an ancestral marine fish, with more extensive body armour, a longer and more streamlined body, and an increased number of gill rakers. Despite reproductive isolation between the two species in the wild[3–6], it is possible to establish productive matings between the two species under laboratory conditions[2]. The resulting F_1 hybrids are viable and fertile, making it possible to carry out a formal genetic analysis of the number and location of loci responsible for the adaptive morphological differences between these naturally occurring vertebrate species.

To develop resources for genome-wide linkage mapping in *Gasterosteus aculeatus*, we used large-scale library screening and sequencing to identify a collection of genomic and complementary DNA clones containing microsatellite repeat sequences. Initially, we sequenced 192 kilobases (kb) of random genomic clones and showed that CA dinucleotides were the most common form of microsatellite in sticklebacks, occurring approximately once every 14 kb. We subsequently screened genomic and cDNA libraries with a $(GT)_{15}$ probe, sequenced 3,560 clones, and identified 1,176 new microsatellite loci. Primers flanking 410 new and 18 previously identified microsatellites[7–9] were designed and used to type a genetic cross between the benthic and limnetic species from Priest Lake,

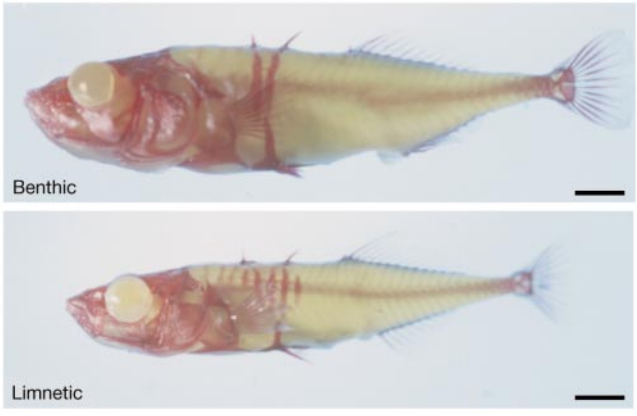

Figure 1 Representative benthic and limnetic fish from Priest Lake, British Columbia, are stained with alizarin red to highlight bone. The benthic fish are larger, more deep-bodied, and have fewer bony lateral plates than the limnetics. Scale bars, 5 mm.

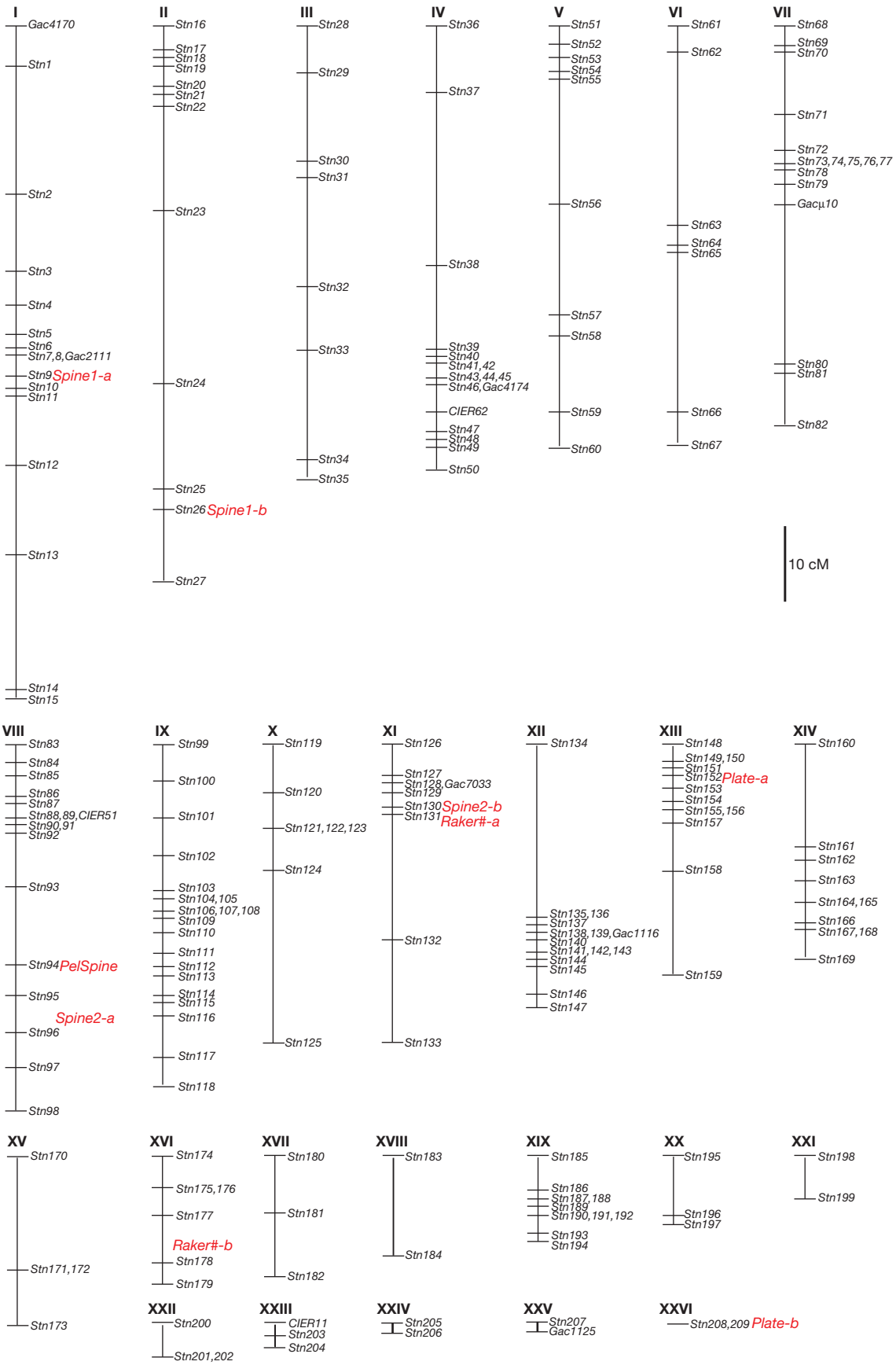

Figure 2 Genetic linkage map of *Gasterosteus aculeatus*. Each linkage group has been assigned a roman numeral (I to XXVI) in order of total genetic length. Microsatellite loci identified at Stanford are designated with a prefix of *Stn*, followed by a number which is based on its serial position in the initial map. Twelve previously published microsatellites are also included on the map[7–9]. The map locations of quantitative trait loci (QTLs) affecting feeding morphology and skeletal armour are shown in red.

British Columbia (Fig. 1). For this cross, an individual Priest benthic female was mated with a single Priest limnetic male, and a single F_1 male (B_1L_1) was crossed to a second Priest benthic female (B_2B_3) to generate 103 progeny. Of the 281 markers that amplified robust bands from the F_1 and benthic parent, 227 (81%) were polymorphic, and therefore informative, in one or both parents. Higher rates of polymorphism were seen in the F_1 male than the benthic female parent (71% versus 57% of 281 markers), consistent with a greater level of genetic diversity between the distinct populations of benthic and limnetic fish than within the benthic population.

The segregation patterns of the 227 informative markers were scored on 92 progeny from the cross, and the 20,884 resulting genotypes were analysed for linkage using JoinMap software[10]. The markers were ordered into 26 linkage groups covering a total genetic distance of 886 centimorgans (cM), using a conservative LOD score (log likelihood ratio) threshold of 4.0 (Fig. 2). *Gasterosteus aculeatus* has a total of 21 chromosomes[11]; therefore, we expect that some current linkage groups will collapse with other groups as additional markers are added to the map. Over 96% of the markers were linked to other markers on the map with an average density of 1 marker per 4 cM, suggesting a high probability that the existing markers can be used for genome-wide linkage mapping of interesting traits in many different stickleback populations.

Previous studies have shown benthic and limnetic species have distinct trophic morphologies adapted to feeding on either small invertebrates in the near shore environment, or zooplankton in the open water[12,13]. Limnetic fish have larger eyes, longer snouts and jaws, and more numerous gill rakers, which are morphological adaptations that enhance feeding performance on small zooplankton[12]. To examine the influence of different genetic regions on trophic morphology, we counted the number of both long and short gill rakers on the first gill arch of all progeny from the Priest Lake cross (Fig. 3b). No major quantitative trait loci (QTLs) were found that influence the number of long gill rakers in the cross consistent with previous biometrical studies suggesting that gill raker number may be based on a large number of genes of small effect[14,15]. In contrast, the number of short gill rakers is influenced by two QTLs that map to separate linkage groups. Together, these two QTLs accounted for nearly two-thirds of the variance in small raker number (Table 1, Figs 2 and 3c). Evolutionary change in the number of short gill rakers may thus be influenced by genetic effects at a relatively small number of chromosome regions.

The amount of skeletal armour is one of the most striking morphological differences between different stickleback populations, including the benthic and limnetic species pairs. Benthics have reduced armour, often with a reduced or absent first dorsal spine, reduced or absent pelvic spines, and a reduced number of lateral plates (Fig. 1). These changes in skeletal armour may be related to the different predation regimes experienced in the near-shore and open-water environments. Open-water populations experience more bird and fish predation, where longer dorsal and pelvic spines appear to offer greater protection[15–17]. In contrast, near-shore populations experience predation by insects, an environment in which spine reduction may be advantageous, and may be accompanied by loss of the lateral plates that support the dorsal and pelvic spines[17,18].

Linkage analysis of spine lengths and lateral plate number in the Priest cross identified QTLs that influence the length of the first and second dorsal spine, the pelvic spine, and the number of lateral plates (Table 1, Figs 2 and 3a, e). These QTLs accounted for 17 to

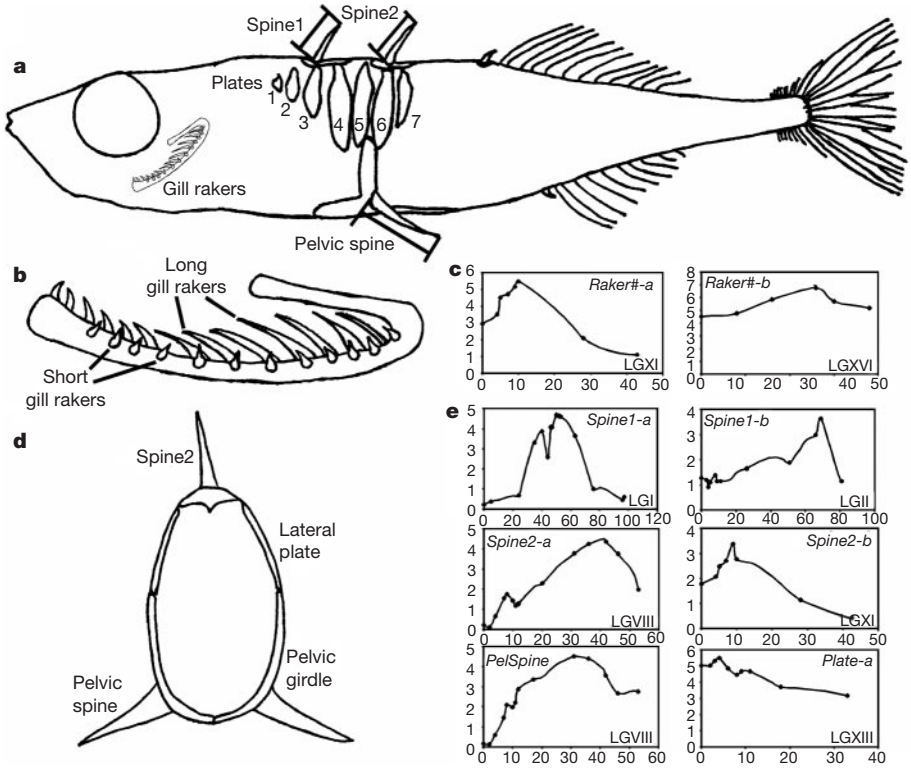

Figure 3 Mapping of morphological traits in the Priest Lake cross. Morphological measurements and traits for which significant QTLs were found: **a**, **d**, Skeletal armour shown in profile (**a**) or cross-section (**d**, adapted from ref. 30); **b**, gill raker numbers. The LOD scores (*y* axis) were graphed relative to position in cM along the linkage group (*x* axis). **c**, Number of small gill rakers; **e**, armour traits. Dots indicate the LOD score for markers on the linkage group. The lines were drawn by plotting the LOD scores calculated by MapQTL at each marker as well as at 5.0-cM intervals along the linkage group. A graph is not shown for the *Plate-b* locus on linkage group XXVI, which consists of two non-recombinant markers.

26% of the total variance in each trait, and mapped to several distinct linkage groups. The locations of the QTLs influencing the first and second dorsal spines were completely distinct, suggesting that very similar morphological features can be influenced by different genetic regions. In contrast, the length of the second dorsal spine and the pelvic spine were both influenced by QTLs that map to a similar region of linkage group VIII. These two spines define the maximal dorsal and ventral extent of stickleback armour (Fig. 3d) and are thought to have an important role in defence against gape-limited predators[15–17]. Variation in the length of these functionally related spines may be due to pleiotroic effects of the same locus or to linked genetic factors on linkage group VIII. Co-localization of ecologically important QTLs has recently been found in other systems, and may influence patterns of naturally occurring variation, adaptation and speciation[19,20].

The variation in length of both the second dorsal spine and the pelvic spine was due primarily to allelic differences segregating within the benthic population (Table 1; see also Supplementary Information). Previous studies have found significant polymorphism for morphological traits within stickleback populations[21], perhaps maintained by spatial or temporal variation in particular habitats, or contrasting advantages of different skeletal phenotypes at different stages of the stickleback life cycle[22,23]. In contrast, the length of the first dorsal spine and number of lateral plates and short gill rakers were influenced by allelic differences both between and within populations (Table 1 and Supplementary Information). Although the presence of a limnetic allele was usually associated with an increase in length or number of skeletal elements, this effect was sometimes seen only in the presence of a particular benthic allele. For example, a limnetic allele at the *Plate-a* QTL was associated with higher mean lateral plate number in combination with the B_3 benthic allele, but not the B_2 benthic allele, suggesting that allelic interactions influence this phenotype (Table 1 and Supplementary Information). For those traits affected by two distinct QTLs, the presence of limnetic alleles at both QTLs often caused a substantial phenotypic effect. For example, fish with 0, 1 or 2 limnetic alleles at the *Plate-a* and *Plate-b* QTL showed an increase from 7.9 to 9.0 to 10.5 in the mean number of lateral plates, and fish with 0, 1 or 2 limnetic alleles at the *Spine1-a* and *Spine1-b* QTL showed an increase from 0.12 mm to 1.0 mm to 1.42 mm in the mean length of the first dorsal spine (Supplementary Information). Therefore, a 33% difference in armour plate number and a greater than tenfold increase in the size of the first dorsal spine can be influenced by genetic effects at a relatively small number of chromosome regions.

Our results in this vertebrate system are consistent with a number of recent genetic studies in plants and insects suggesting that evolutionary changes between organisms are controlled by genes with a variety of magnitudes of effect, some of which account for a substantial fraction of the variance in particular traits[24]. The small size of the current cross limits our ability to detect additional genes of smaller effect, and could overestimate the contribution of minor QTL[25]. Larger crosses are now needed to identify the full spectrum of genetic changes that contribute to the morphological differences between benthic and limnetic fish, and to narrow the location of the gene or genes within each chromosome interval that contribute to morphological divergence.

The many different chromosome regions that affect specific aspects of skeletal anatomy in sticklebacks reveal a flexible genetic system for independent modification of the size and number of different feeding and armour structures. However, some functionally related traits map to similar chromosome regions, suggesting that genetic linkage or pleiotropy may help account for the covariation in dorsal and pelvic spine lengths previously reported in many stickleback populations[15]. An extensive literature already exists on the great variation in size, morphology, colour and behaviour of freshwater sticklebacks around the world[1]. The ease of collecting and crossing such fish in the laboratory, the relatively compact genome size of sticklebacks[26], the development of genome-wide linkage maps, and the ability to detect QTL for important evolutionary differences provide a particularly favourable system for further molecular studies of the genetic basis of morphological and behavioural changes during vertebrate evolution. □

Methods

Library construction, screening and sequencing

Genomic DNA from a single Paxton lake adult fish was digested with *Rsa*I and fragments from 0.8–1.6 kb were cloned into the *Eco*RV site of pBluescriptSK(+). An oligo(dT) primed cDNA library was constructed in lambda ZAP Express (Stratagene) using RNA isolated from the head and internal organs of two adult fish from Salinas River, California. Colony and phage lifts were hybridized with an end-labelled oligonucleotide $(GT)_{15}$ in 4 × SSPE, 1 × Denhardt's solution, and 1% SDS at 60 °C overnight, and then washed for 2 × 10 min in 0.1 × SSC and 0.1% SDS at 60 °C. All positive clones were grown overnight in 96-well plates, and plasmid DNA was prepared using the QIAprep96 Turbo miniprep (Qiagen). Each clone was sequenced with a T3 and a T7 primer on a 96-lane gel on an ABI377 sequencer. Clones containing a microsatellite repeat were analysed with Primer3 (ref. 27) to identify primer pairs flanking the microsatellite. Primer pairs were picked to have melting temperatures between 55–65 °C and to give products between 100–250 bp. Forward primer plates were labelled with one of three 5′ phosphoramidite fluorescent conjugates: 6-fam (blue), 6-hex (yellow) or tet (green).

Table 1 Location and magnitude of effect for QTLs

Trait	Locus name	Linkage group		LOD	PVE (%)	Phenotype means		
					L_1B_2	B_1B_2	L_1B_3	B_1B_3
Feeding modifications								
Gill raker numbers	*Raker#-a*	XI	5.5*	26	15.4	14.3	13.8	13.8 rakers†‡§
	Raker#-b	XVI	6.8*	37	14.9	13.2	14.4	14.3 rakers†§
Body armour								
Lateral plate number	*Plate-a*	XIII	5.5*	26	8.5	8.6	10.6	8.4 plates†‡§
	Plate-b	XXVI	4.6*	22	10.0	8.2	9.6	8.0 plates†
Dorsal spine 1 length	*Spine1-a*	I	4.7*	21	1.53	0.49	1.00	0.57 mm†
	Spine1-b	II	3.6	17	1.42	0.73	0.97	0.13 mm†‡
Dorsal spine 2 length	*Spine2-a*	VIII	4.5*	22	2.72	2.59	2.31	2.35 mm‡
	Spine2-b	XI	3.4	17	2.57	2.65	2.22	2.39 mm‡
Pelvic spine length	*PelSpine*	VIII	4.5*	25	3.35	3.41	2.91	2.84 mm‡

For each QTL detected, the linkage group maximum LOD score, and percentage of the phenotypic variance explained (PVE) are indicated. QTLs were scored as significant or suggestive based on conservative recommendations for genome-wide linkage mapping[29], and by permutation testing for each trait. The genome-wide LOD significance thresholds are 4.2 for gill raker number and pelvic spine length, and 4.3 for plate number and dorsal spine length. The chromosome-wide LOD suggestive thresholds are 2.9 for length of dorsal spine 1 on linkage group II, and 2.6 for length of dorsal spine 2 on linkage group XI. Mean phenotypic values of each trait were also calculated for those progeny that inherited either all benthic alleles (B_1B_2 and B_1B_3), or both limnetic and benthic alleles (L_1B_2 and L_1B_3) at the most closely linked microsatellite. Significant differences between phenotype means are the estimated effect of alternate alleles inherited from the F_1 male parent (L_1 or B_1, the between-species effect), alleles inherited from the benthic female parent (B_2 or B_3, the within-species effect) and/or their interaction. See Supplementary Information for details.

* Significant QTLs.

† Between-species effect.

‡ Within-species effect.

§ Interaction between alleles from L_1 or B_1 and B_2 or B_3.

Genotyping

All polymerase chain reactions were carried out in a PTC-200 DNA Engine thermocycler (MJ Research) in 10-μl reactions containing 0.5 μM of each primer, 5 ng DNA, 0.25 mM dNTPs (Pharmacia), 1.5 mM $MgCl_2$, and 0.25 units Taq polymerase (PE Applied Biosystems). The cycling conditions for all primer pairs were 1 cycle of 95 °C for 1 min 45 s, 56 °C for 45 s and 72 °C for 45 s; 5 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s; and 30 cycles of 90 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s, followed by a final cycle of 72 °C for 5 min. PCR products from 3 to 6 different primer pairs were then pooled and analysed on a 96-lane gel on an ABI377 with Gene Scan 2.1 software (Applied Biosystems) and GENESCAN-500 TAMRA (Applied Biosystems) used as internal size standard.

Linkage map construction

A genetic linkage map was created using JoinMap version 2.0 (ref. 10) on a locus file containing genotypes of 227 microsatellite loci in 92 backcross progeny, with the population type set for segregation of up to four alleles per locus (cross-pollinator). The JMGRP module of JoinMap was used with a LOD score threshold of 4.0 to assign 219 of the 227 loci to 26 linkage groups. The JMREC module was then used on each of the linkage groups to determine phase information for each locus. For each linkage group, a map was created with the JMMAP module: Kosambi mapping function, LOD threshold of 0.001, recombination threshold of 0.499, jump threshold of 5.0, triplet value of 5.0, and no fixed order. A ripple was performed after all markers on the linkage group were added to the map.

Morphological analysis

Fish were fixed in 10% buffered formalin for at least 1 week, placed in distilled H_2O (dH_2O) for 24 h, stained with 0.008% alizarin red in 1% KOH for 24 h, placed in dH_2O for 24 h, and placed in 37% isopropyl alcohol for final storage. Measurements were done with Vernier callipers accurate to 0.02 mm. Lateral plates were counted on both sides of the body, and the number of long and short gill rakers were counted on the left side of the first gill arch.

QTL mapping

All morphological traits were analysed with MapQTL 3.0 (ref. 28) using the interval mapping method which fits a single QTL model that is based on four possible segregating genotypes and does not assume a particular model of relationship between benthic and limnetic alleles. The parameters used were: mapping step size of 5.0, maximum of 200 iterations, and functional tolerance value of $1.0e^{-8}$. A maximum of five flanking markers were used to resolve incomplete genotypes. Significance thresholds for linkage were chosen using conservative criteria for genome-wide linkage mapping in non-inbred individuals: suggestive linkage of LOD ≥ 3.2, significant linkage of LOD ≥ 4.5 (ref. 29). Significance thresholds were confirmed by permutation tests in MapQTL 4.0, with a genome-wide significance level of $\alpha = 0.05$, $n = 1{,}000$ for significant linkages and a chromosome-wide significance level of $\alpha = 0.05$, $n = 1{,}000$ for suggestive linkages. Suggestive loci were only reported for those traits which were also influenced by one or more significant QTL. Multiple QTL model (MQM) mapping with initial QTL did not change the results. Calculation of the percentage of phenotypic variance explained by a QTL was done in MapQTL 3.0 on the basis of the population variance found within the progeny of the cross.

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Competing interests statement

The authors declare that they have no competing financial interests.

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