Modular Skeletal Evolution in Sticklebacks Is Controlled by Additive and Clustered Quantitative Trait Loci

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ABSTRACT Understanding the genetic architecture of evolutionary change remains a long-standing goal in biology. In vertebrates, skeletal evolution has contributed greatly to adaptation in body form and function in response to changing ecological variables like diet and predation. Here we use genome-wide linkage mapping in threespine stickleback fish to investigate the genetic architecture of evolved changes in many armor and trophic traits. We identify >100 quantitative trait loci (QTL) controlling the pattern of serially repeating skeletal elements, including gill rakers, teeth, branchial bones, jaws, median fin spines, and vertebrae. We use this large collection of QTL to address long-standing questions about the anatomical specificity, genetic dominance, and genomic clustering of loci controlling skeletal differences in evolving populations. We find that most QTL (76%) that influence serially repeating skeletal elements have anatomically regional effects. In addition, most QTL (71%) have at least partially additive effects, regardless of whether the QTL controls evolved loss or gain of skeletal elements. Finally, many QTL with high LOD scores cluster on chromosomes 4, 20, and 21. These results identify a modular system that can control highly specific aspects of skeletal form. Because of the general additivity and genomic clustering of major QTL, concerted changes in both protective armor and trophic traits may occur when sticklebacks inherit either marine or freshwater alleles at linked or possible "supergene" regions of the stickleback genome. Further study of these regions will help identify the molecular basis of both modular and coordinated changes in the vertebrate skeleton.

UNDERSTANDING the quantitative genetic architecture underlying evolutionary change in nature remains a major goal in genetics. The past two decades have seen a rapid increase in experimental data from various model systems, generating vigorous debate over the relative importance of coding *vs.* regulatory alleles, the prevalence of pleiotropy, and the role of large-effect mutations during adaptation to new environments (Stern and Orgogozo 2008; Streisfeld and Rausher 2011; Rockman 2012).

One particularly interesting genetic architecture found in several natural systems is close linkage of loci controlling multiple, often coadaptive, phenotypes. Such trait clusters, sometimes called "supergenes," have been observed in primroses (Darwin 1877; Mather 1950; Li *et al.* 2011), butterflies (Clarke *et al.* 1968; Mallet 1989; Joron *et al.* 2006), snails (Murray and Clarke 1976), and fish (Winge 1927; Protas *et al.* 2008; Roberts *et al.* 2009; Tripathi *et al.* 2009). Trait clusters could result from recombination suppression (Noor *et al.* 2001), for example through chromosomal inversions (Lowry and Willis 2010; Joron *et al.* 2011; Fishman *et al.* 2013). Alternatively, trait clusters could result from tightly linked loci or pleiotropic effects of individual genes (Mallet 1989; Studer and Doebley 2011). Having

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multiple different phenotypes controlled by the same genomic region could greatly facilitate rapid adaptive evolution (Kirkpatrick and Barton 2006; Feder *et al.* 2011; Yeaman and Whitlock 2011).

Adaptive mutations may arise *de novo* or be selected from preexisting standing variants that become favorable following environmental change. When selection acts on newly arising mutations, dominant alleles should have a higher probability of fixation than recessive alleles (Haldane 1927). However, if previously unfavorable standing variant alleles become advantageous following environmental change, there is little bias in the likelihood of alleles of different dominances to sweep to fixation (Orr and Betancourt 2001). Therefore in systems where selection from standing variation predominates, the observed distribution of dominances should largely reflect the underlying distribution of dominances of advantageous mutations. Although most new mutations are recessive (Fisher 1928; Orr 1991), advantageous mutations may have a different distribution of dominances than all mutations. Dominance distributions of adaptive mutations are still poorly characterized, particularly for alleles underlying morphological traits in natural vertebrate populations.

Cis-regulatory changes may predominate during morphological evolution because of the highly pleiotropic effects of developmental regulatory genes (Stern 2000; Carroll 2008). Protein-coding changes in such genes will alter the gene's function at all sites of expression. In contrast, *cis*-regulatory changes can alter expression at highly specific times or locations, limiting phenotypic effects to subdomains of a gene's function. This idea predicts that quantitative trait loci (QTL) controlling adaptive morphological changes may typically act in subsets of anatomical regions. Although this idea can be tested by looking for regional *vs.* global effects among evolutionary QTL that influence serially repeating morphology, few studies have examined large numbers of traits to test the prevalence of modular genetic effects in naturally evolved species (Wagner *et al.* 2007).

The threespine stickleback (*Gasterosteus aculeatus*) species complex provides a powerful system for forward genetic dissection of repeated evolution in nature. Migratory marine sticklebacks colonized thousands of new freshwater lakes and streams following the last Ice Age. Newly established freshwater populations evolved similar phenotypes in response to similar ecological conditions, providing strong evidence that the corresponding traits evolve by natural selection (Schluter 2000). Despite dramatic morphological and physiological differences among sticklebacks, intercrosses between populations produce viable and fertile offspring, making it possible to study the genetic and genomic mechanisms that underlie adaptive evolution in new environments (reviewed in Kingsley and Peichel 2007; Schluter *et al.* 2010). The remarkably compact genome size (~460 Mb) has facilitated a high-quality genome assembly and resequencing of fish from 20 different populations, revealing abundant reuse of standing variants as one of several mechanisms underlying evolutionary differences in this system (Jones *et al.* 2012b).

Previous studies have identified many trophic and defensive armor traits that evolve repeatedly in freshwater (Bell and Foster 1994). A classic case of ecology-driven natural selection is the reduction in number of gill raker bones (Schluter 2000) in countless freshwater stickleback populations throughout the northern hemisphere (Hagen and Gilbertson 1972; Gross and Anderson 1984). Oceanic fish primarily feed on tiny zooplankton in the water column, while freshwater fish adapted to the benthic zone (bottom of lake) have shifted to a diet of larger invertebrates living in sediments or attached to vegetation (Kislalioglu and Gibson 1977; Gross and Anderson 1984). Both reduced gill raker number and larger jaw gape are found in benthic-adapted species (Schluter and McPhail 1992). While large jaws and low gill raker counts correlate with more successful benthos foraging (Lavin and McPhail 1986), small jaws and high gill raker counts correlate with more successful foraging of small prey from the water column (Bentzen and McPhail 1984). Benthic-adapted stickleback forms also display changes in skull morphology that distinguish them from forms adapted to eat smaller prey items (Willacker *et al.* 2010; McGee *et al.* 2013). Collectively, these studies suggest that a concerted set of craniofacial changes allows freshwater populations to forage more efficiently on new diets in freshwater habitats. In addition to head skeletal traits, aspects of the median fin and vertebral skeleton are known to vary and be under selection in stickleback populations. These include dorsal spine lengths (Gross 1978; Bell *et al.* 2006; Hunt *et al.* 2008), the number and position of dorsal and anal fin rays and their supporting pterygiophores, and vertebral number and positioning (Swain 1992a,b; Ahn and Gibson 1999).

Here we apply genome-wide linkage mapping to investigate the genetic architecture of >100 trophic, armor, and serially repeating skeletal traits in sticklebacks. Using a large set of newly identified QTL, we address several general questions in evolutionary genetics, including the extent to which loci are clustered in the genome, the dominance distribution of evolutionary alleles, and the proportion of loci that have anatomically regional effects. Our results show that loci controlling both regressive (loss) and constructive (gain) traits are clustered in the stickleback genome, making it possible to shape multiple aspects of both trophic and defensive morphology by co-inheritance of marine or freshwater alleles at linked loci.

Materials and Methods

Ethics statement

All animal work was approved by University of British Columbia and Stanford University Institutional Animal Care and Use Committees (protocols A97-0298 and 13834).

QTL mapping

A family of 370 full-sibling F_2 fish derived from a Japanese Pacific marine grandmother and a Paxton Lake (British Columbia, Canada) benthic freshwater grandfather (Colosimo *et al.* 2004) was genotyped with 275 microsatellite markers

and phenotyped for 110 skeletal traits across eight trait classes. Linkage map construction, skeletal phenotype analyses, trait transformations, significance threshold determinations, and QTL mapping using R/qtl (Broman and Sen 2009) are described in Supporting Information, File S1. New microsatellite markers are listed in Table S1. QTL within the same trait class with overlapping 1.5-LOD intervals were filtered, keeping the QTL with the highest LOD and removing lesser-effect QTL to avoid redundant QTL sampling. This filtered QTL set was used for all dominance and clustering analyses. All raw phenotype, adjusted phenotype (see Table S2), and genotype data used for QTL mapping are presented in File S2. Details on the genetic positions, effect sizes, and dominances of all QTL are presented in File S3 and File S4.

Anatomical specificity of QTL

For investigating the anatomical specificity of QTL, the subset of QTL with clearly or likely serially homologous domains (QTL controlling gill raker number, pharyngeal tooth number, branchial bone length, upper and lower jaw size, and dorsal spine lengths) was considered. QTL were considered regional if they affected a subset of domains and global if they affected all domains.

Dominance analyses

To calculate the dominance of each QTL, *Z*-scored residual phenotypes were first calculated from a linear regression of the phenotype against all other peak marker genotypes affecting that phenotype. For calculating dominance, the equation d/a (Falconer 1989) was used, with a representing the additive effect of one additional benthic allele (*i.e.*, half the phenotypic difference between the homozygous benthic and homozygous marine genotypic classes). d represents the dominance effect: the difference between the heterozygous phenotype and the midpoint between homozygous parental phenotypes. Similar to a sunflower domestication QTL study (Burke *et al.* 2002), we used the following d/a ranges to classify the dominance effect of benthic alleles: < -1.25 for underdominant, -1.25 to -0.75 for recessive, -0.75 to -0.25 for partially recessive, -0.25 to 0.25 for additive, 0.25 to 0.75 for partially dominant, 0.75 to 1.25 for dominant, and >1.25 for overdominant. For each QTL, one value of a and two values of d were calculated by Haley–Knott regression, as two classes of heterozygous F_2 animals were present. For 2 of 342 QTL, the chromosome had only one heterozygous genotypic class (because F_1 parents had same heterozygous genotypes across this chromosome). For these 2 QTL, the single value of d was counted twice. For the other 340 QTL, d for both the M_1B_1 and M_2B_2 (M, marine; B, benthic) heterozygous genotypic classes was calculated. Simulations investigating dominance bias in detection, *i.e.*, whether additive QTL were less likely to be detected than recessive or dominant QTL, are described in File S1.

Tests of trait clustering

To determine whether QTL were significantly clustered in the genome, we took the observed number of QTL per trait class for (1) all QTL or (2) large-effect QTL, defined as the top quartile of QTL by LOD score (LOD $>$ 8.95, see File S1). We then simulated 1000 random placements of peak markers in the genome, allowing only one QTL per trait class per chromosome (similar to the QTL filtering method described above). For each simulation, we determined the number of QTL with peak markers $<$5 cM away. We calculated *P*-values by comparing the observed number of QTL having peak markers within 5 cM to this null distribution. To determine whether the number of QTL on a single chromosome was significantly enriched relative to a null hypothesis of independent and evenly distributed QTL, simulations were also performed with all of the QTL or the top quartile of QTL by LOD score. For each simulation, the observed total number of trait classes with QTL on each chromosome was determined. This observed set of QTL for each trait class was distributed randomly to chromosomes without replacement with probability in proportion to (1) the genetic length of the chromosome, (2) the physical length of the chromosome (Jones *et al.* 2012b), or (3) the number of Ensembl-predicted genes (Jones *et al.* 2012b) within the chromosome. For each case, 10,000 simulations were performed to calculate a null distribution of QTL per chromosome as well as a mean number of "expected" QTL. For every chromosome, the true number of QTL was compared to the null distribution to calculate a *P*-value. Since sexually dimorphic traits represent a genetic effect of the sex chromosome (chromosome 19) and this effect was largely statistically removed prior to QTL mapping, chromosome 19 was excluded from the clustering simulations and analysis.

Results

Major skeletal differences between marine and freshwater fish

The skeletons of Paxton benthic freshwater (PAXB) sticklebacks show multiple obvious reductions in external bones compared to Japanese Pacific marine (JAMA) animals, including reduced size and number of armor plates, loss of pelvic fins, and reduced length of dorsal spines (Figure 1, A and B). In addition, computerized tomography (Figure 1, C–H) revealed a mixture of both regressive ("loss") and constructive ("gain") traits in the skull and internal branchial skeleton of PAXB sticklebacks. The derived freshwater fish show dramatic reductions in the number and length of gill rakers (Figure 1, C and D), as expected based on previous studies (McPhail 1992; Kitano *et al.* 2007). However, PAXB branchial bones, especially the first epibranchial, have increased in length compared to their marine counterparts (arrowheads in Figure 1, C and D). In addition, PAXB fish have roughly twice the number of ventral pharyngeal teeth seen in JAMA animals (Figure 1, E and F). Compared to marine animals, PAXB fish also show a longer and thinner supraoccipital crest, a posterior process on the supraoccipital bone at the back of the skull that serves as the insertion point for muscles involved in buccal cavity opening (Figure 1, G

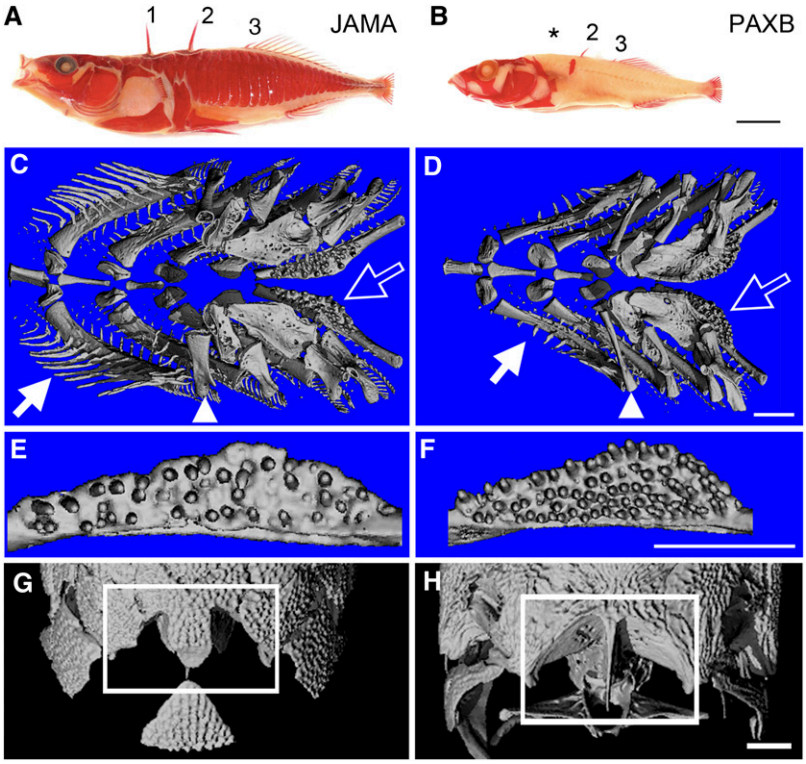

Figure 1 Evolved skeletal differences between marine and benthic sticklebacks. Skeletal morphology was revealed by Alizarin red staining (A and B) or microcomputerized tomography (C–H) of adult Japanese Pacific marine (JAMA) (A, C, E, and G) and Paxton benthic freshwater (PAXB) (B, D, F, and H) fish. (A and B) Lateral views of bone-stained adults reveal differences in dorsal spine lengths. Three dorsal spines are numbered in A. The first dorsal spine is missing (asterisk) in this PAXB fish. (C and D) Dorsal views of branchial skeletons reveal fewer and less densely spaced gill rakers (white arrows) and longer branchial bones (white arrowheads) in PAXB. (E and F) Ventral pharyngeal toothplates (labeled with open white arrow in C and D) reveal higher tooth number in PAXB. (G and H) Dorsal views of skulls reveal differences in the size and shape of the supraoccipital crest (white boxes). The JAMA supraoccipital crest is shorter and wider but larger in area, while the PAXB supraoccipital crest is longer and narrower, smaller in area, and flanked by more robust insertion points for the epaxial muscles. Bars, 1 mm.

and H). Increased size of these muscles is a characteristic feature of PAXB fish, and is thought to increase force generation and suction pressure for feeding on attached littoral prey items (McGee *et al.* 2013).

To investigate the genetic architecture underlying the evolution of these trophic, armor, and other skeletal traits, we used genome-wide linkage mapping in a large marine × benthic F_2 genetic cross previously studied for lateral plate, pelvic spine, and pigmentation patterning (Colosimo *et al.* 2004, 2005; Shapiro *et al.* 2004; Miller *et al.* 2007). We phenotyped 370 full-sibling F_2 fish from this cross and then mapped QTL influencing 110 different skeletal phenotypes (Table S2), including a large number of traits in the branchial skeleton (Figure 2, A–I); multiple aspects of jaw, skull, and opercle morphology (Figure 2, J–O); dorsal and anal spine lengths and degree of spine serrations (Figure 2, P and Q); and several median fin and vertebral traits (Ahn and Gibson 1999) proposed to be important for freshwater adaptation (Figure 2Q).

Most traits are sexually dimorphic

As most traits (93 of 110) are size and/or sex dependent, we systematically corrected for size and/or sex (Table S2). In total, 72 traits showed significant differences between the sexes (Table S3). The traits that show dimorphism, and the direction of dimorphism, were largely consistent with previous reports. For example, males had bigger jaws, more oral teeth, more vertebrae, fewer abdominal vertebrae, more dorsal and anal fin rays, more pterygiophores, and dorsal spines with more serrations both in the current study and in previous studies (Lindsey 1962; Reimchen and Nelson 1987; McPhail 1992; Caldecutt *et al.* 2001; Kitano *et al.* 2007, 2009).

Most QTL are anatomically specific

Raw or corrected trait values (Table S2) and a genome-wide linkage map (Figure S1) were used to map QTL with a multiple-QTL mapping approach in R/qtl (Broman and Sen 2009). This analysis (described in File S1) identified 342 total QTL for 92 of the 110 traits (File S3). Based on segmental homology and likely embryonic origin, we divided the 110 skeletal traits into eight trait classes: rakers, teeth, branchial bones, jaw, skull, opercle, median fin, and vertebrae (Figure 3). In cases where a particular chromosome region had an effect on multiple phenotypes within the same trait class, it is parsimonious to assume that a single underlying QTL affected the trait across several domains. Thus, for our analysis of the properties of these QTL, we conservatively considered such QTL only once. To define a minimally nonredundant set of QTL by trait class, we included only QTL whose 1.5-LOD intervals [an ~95% confidence interval (Dupuis and Siegmund 1999)] did not overlap. This filtering defined a set of 118 QTL across the eight trait classes (Table S4, Figure 3). The overall distribution of percentage of variance explained (PVE) of these QTL consisted of many small-effect QTL and few large-effect QTL (Figure S2), similar to that in a previous stickleback shape QTL study (Albert *et al.* 2008). For trait classes that had multiple serially homologous anatomical domains or elements (raker number, teeth, branchial, jaw, and spine classes), we asked whether QTL controlling these

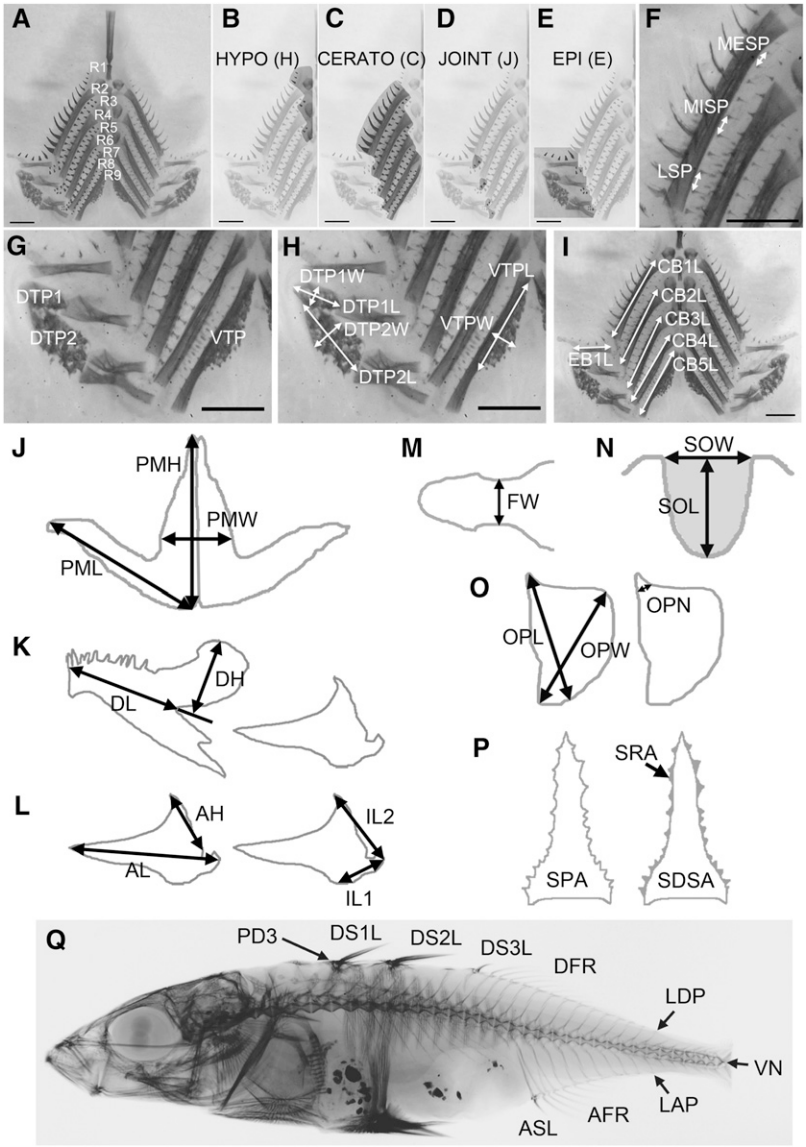

Figure 2 Raker, tooth, branchial bone, jaw, skull, opercle, median fin, and vertebral skeletal phenotypes. (A–I) Branchial skeleton from Alizarin red-stained F_2 fish, dissected and flattened into two-dimensional preparation by single incision along the dorsal midline. All branchial bones, including pharyngeal toothplates and gill rakers, are readily visible. Bars, 1 mm. (A) Nine rows of gill rakers (R1–R9), pseudocolored black on left half, line the anterior and posterior faces of each segment, except for the last segment, which lacks gill rakers on its posterior side. (B–E) Dorsal–ventral domains of gill rakers, defined by edges of branchial bones (see File S1) from ventromedial to dorsal: hypo (B), cerato (C), joint (D), and epi (E) gill raker domains. (F) Three interraker spacing measurements in lateral (LSP), middle (MISP), and medial (MESP) regions of row 2 cerato rakers. (G) Three pharyngeal toothplates (two dorsal and one ventral) are present on each side. (H) Pharyngeal toothplate lengths and widths. (I) Branchial bone lengths. (J) Dorsal view of premaxilla bone (upper jaw) traits. (K) Lateral view of dentary bone (lower jaw, shown with articular posteriorly). (L) Lateral view of articular bone traits. (M) Dorsal view of frontal bone width (or interorbital distance). (N) Dorsal view of caudal end of supraoccipital bone, with supraoccipital crest area shaded gray (also see Figure 1, G and H). (O) Lateral view of opercle bone traits. (P) Second dorsal spine area and serration traits. (Q) X ray showing spine, median fin ray, and vertebral position landmarks. Abbreviations (defined in Table S2): AFR, number of anal fin rays; AH, articular height; AL, articular length; ASL, anal spine length; CB1L, ceratobranchial 1 length; CB2L, ceratobranchial 2 length; CB3L, ceratobranchial 3 length; CB4L, ceratobranchial 4 length; CB5L, ceratobranchial 5 length; DFR, dorsal fin ray number; DH, dentary height; DL, dentary length; DS1L, dorsal spine 1 length; DS2L, dorsal spine 2 length; DS3L, dorsal spine 3 length; DTP1, dorsal toothplate 1; DTP1L, dorsal toothplate 1 length; DTP1W, dorsal toothplate 1 width; DTP2, dorsal toothplate 2; DTP2L, dorsal toothplate 2 length; DTP2W, dorsal toothplate 2 width; EB1L, epibranchial 1 length; FW, frontal width; IL1, in-lever 1 of the articular; IL2, in-lever 2 of the articular; LAP, vertebrae number of last anal pterygiophore; LDP, vertebrae number of last dorsal pterygiophore; LSP, lateral row 2 raker spacing; MESP, medial row 2 raker spacing; MISP, middle row 2 raker spacing; OPL, opercle length; OPN, opercle neck width; OPW, opercle width; PD3, vertebrae number of third predorsal pterygiophore; PMH, premaxilla height; PML, premaxilla length; PMW, premaxilla width; R1–R9, rows 1–9 of gill rakers; SDSA, smoothened dorsal spine 2 area; SOL, supraoccipital crest length; SOW, supraoccipital crest width; SPA, dorsal spine 2 area; SRA, spine 2 serration area; VN, total vertebrae number; VTP, ventral toothplate; VTPL, ventral toothplate length; VTPW, ventral toothplate width.

traits had anatomically regional or global effects. Of this set of QTL, a large majority (76%) affected only a subset of the possible domains while 24% affected all domains (Figure 4, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7, Table S5).

Gill rakers: Gill rakers are present in nine rows from anterior to posterior and in four regions from dorsal to ventral (Figure 2, A–E). Given well-established regional developmental genetic control of pharyngeal segments along the anterior/posterior (A/P) and dorsal/ventral (D/V) axes (*e.g.*, *Hox* gene control of segmental identity and *Dlx* gene control of D/V patterning, reviewed in Minoux and Rijli 2010), we scored and mapped gill raker number separately for each individual A/P and D/V domain, as well as composite phenotypes that represented putative developmental domains [*e.g.*, all ventral (cerato) rakers]. Overall, we found 23 QTL controlling gill raker number or spacing. The raker QTL displayed a high degree of regional specificity, with no QTL having significant effects in all possible domains (Table S5, Figure 4). For example, the largest-effect gill raker QTL mapped to chromosome 20 and had strictly ventral (cerato)-specific effects on raker number, while chromosomes 10 and 7 had regional effects on joint and hypo rakers, respectively (Figure 4 and Figure S3). Additionally, on chromosome 4, the second-largest-effect raker QTL mapped to one end of the chromosome and was largely ventral (cerato)-specific, whereas a nonoverlapping region more centrally located on chromosome 4 had a dorsal (epi)-specific effect on raker number (see below). Both chromosome 4 and chromosome 20 large-effect gill raker number QTL controlled gill raker number at least in

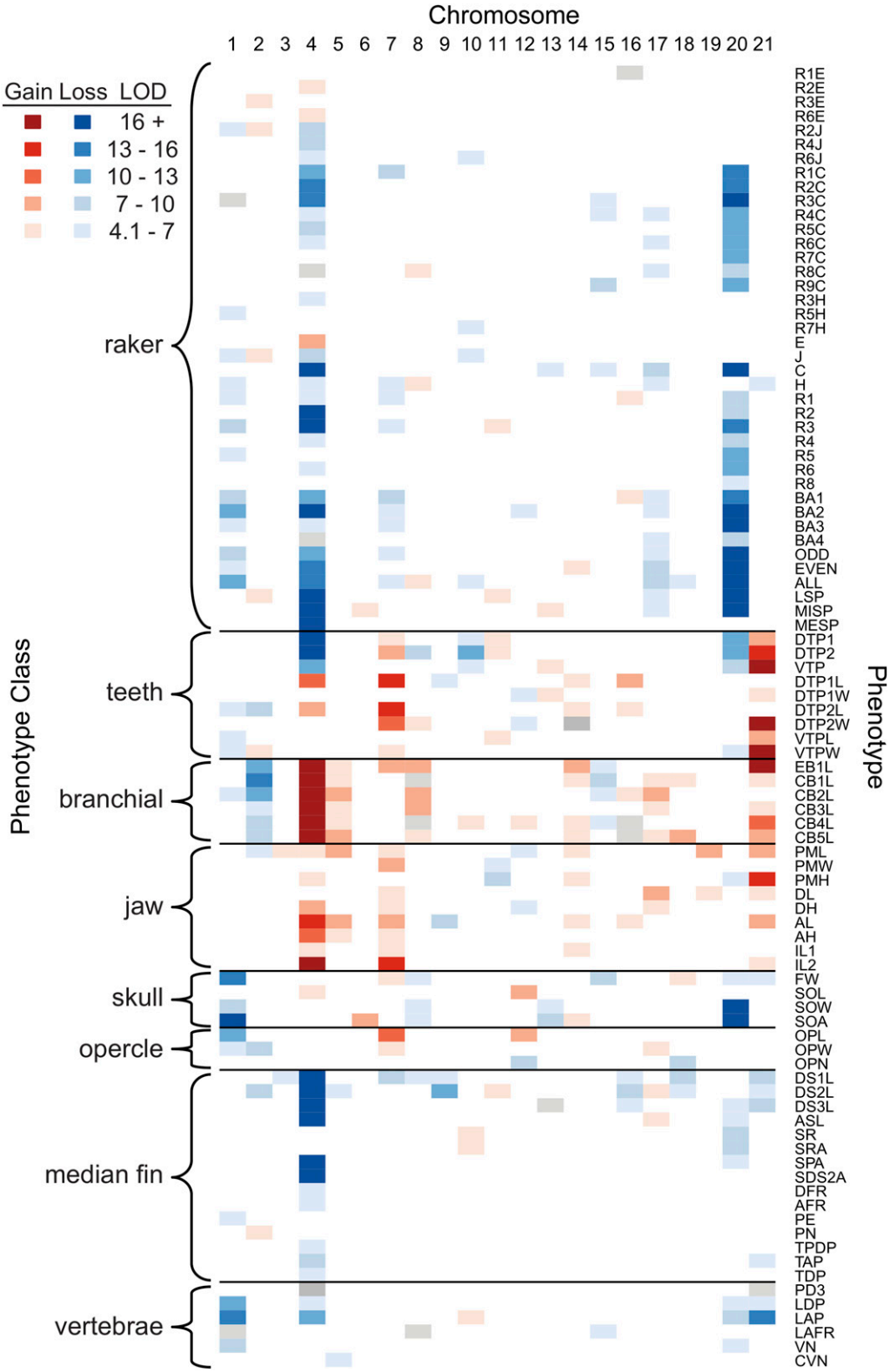

Figure 3 Genome-wide overview of detected skeletal QTL. Classes of traits are grouped on the left and individual traits listed on the right. Abbreviations are defined in Figure 2 legend and in Table S2. For each trait, the LOD score for each QTL on each chromosome is indicated by the heat map shown at the top left, with "gain" traits (benthic allele confers more or bigger bones) colored red and "loss" traits (benthic allele confers fewer or smaller bones) colored blue. Heterotic QTL (homozygous marine and benthic F_2 fish do not differ significantly in phenotype by two-tailed *t*-test) and vertebral position QTL (trait is neither loss nor gain) are shaded gray.

part through controlling gill raker spacing, as row 2 interraker spacing mapped strongly to an overlapping region of chromosomes 4 and 20. Four raker QTL (right chromosome 4 and chromosomes 6, 11, and 13) had a raker spacing measurement map more strongly than any raker number trait.

Teeth: We identified 20 QTL on 14 chromosomes controlling tooth number or toothplate size (Figure 2, G and H), including two QTL with large effects on tooth number (Table S4). A QTL on chromosome 21 explained nearly a third of the variance in ventral pharyngeal tooth number, while

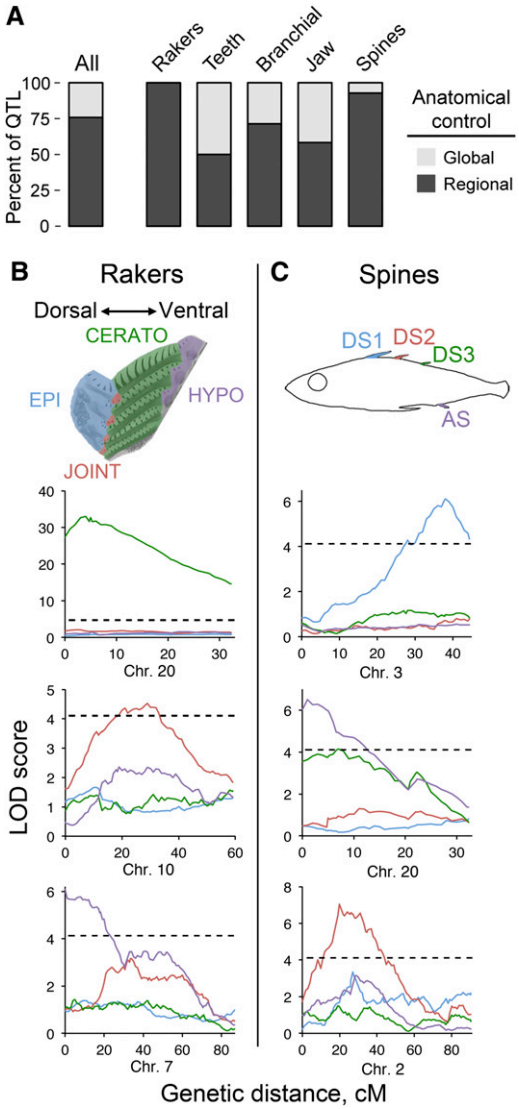

Figure 4 Skeletal QTL with anatomically regional effects. (A) Proportion of QTL with regional *vs.* global effects. For trait classes containing multiple serial or developmental homologous elements or domains, percentages of QTL controlling some (black) or all (gray) elements or domains within the trait class are shown. (B and C) Gill raker (B) and median fin spine (C) morphologies are controlled mainly by QTL with highly regional effects. (B, top) Color-coded dorsal–ventral domains of gill rakers. From dorsal to the ventral midline, rakers are present in EPI (dorsal, blue), JOINT (intermediate, red), CERATO (ventral, green), and HYPO (ventromedial, purple) domains. Below, examples of three gill raker QTL with regional effects in these dorsal–ventral domains are shown, from top to bottom: chromosomes 20, 10, and 7 have regional cerato (ventral), joint, and hypo-specific domains, respectively. Raker totals in each domain are mapped separately and results are color coded as in the raker schematic. (C, top) Three dorsal spines (DS1–DS3) and one anal spine are color coded in the spine schematic. Below are examples of three spine QTL on chromosomes 3, 2, and 20 having regional effects on DS1, DS3 + AS, and DS2 respectively. In each QTL plot, genetic distance in centimorgans is on the *x*-axis and LOD score on the *y*-axis. Dashed lines are significance thresholds.

a QTL on chromosome 4 had large effects on dorsal pharyngeal tooth number. All tooth number QTL had stronger effects (>2 LOD units difference) on either dorsal or ventral pharyngeal tooth number (Figure S4). Genetic control of oral and pharyngeal tooth variation appeared largely independent. Although oral tooth number in the upper jaw was sexually dimorphic (Table S3) as previously reported for several wild stickleback populations (Caldecutt *et al.* 2001), no autosomal QTL for oral tooth number were detected (Table S2).

Branchial bones: We measured the lengths of all five ceratobranchials (large ventral bones in the branchial skeleton), as well as the first epibranchial, a dorsal branchial bone (Figure 2I). We identified 16 QTL on 14 chromosomes controlling branchial bone length, including two QTL on chromosomes 4 and 21 that had large effects on ventral and dorsal bones, respectively (Table S4, Figure S5). QTL with anatomically regional or broad effects were both detected in the cross: four QTL (chromosomes 1, 7, 10, and 12) had significant effects on only one of six bones analyzed, while 4 other QTL (chromosomes 2, 4, 5, and 8) had significant effects on all six branchial bones analyzed.

Jaw: Unlike the branchial bones, which ossify endochondrally, the premaxilla and dentary, the major bones of the fish upper and lower jaw (Figure 2, J–L), respectively, are dermal bones that form without a cartilage intermediate (Anker 1974; Cubbage and Mabee 1996). We detected 15 QTL on 14 chromosomes controlling jaw morphology (Table S4, Figure S6). Eleven QTL influenced the size of the premaxilla, with the largest-effect QTL on chromosome 21. Ten QTL influenced the size of the dentary and the associated articular bone, with the largest-effect QTL mapping to chromosome 4. Three QTL (chromosomes 2, 3, and 20) controlled upper but not lower jaw size and 3 other QTL (chromosomes 9, 16, and 17) controlled lower but not upper jaw size (Figure S6). In contrast, 7 QTL (chromosomes 4, 5, 7, 12, 14, 19, and 21) had significant effects on both the upper and lower jaw.

Skull: In the posterior skull, the supraoccipital crest is longer and thinner in benthic fish than in marine fish (Figure 1, G and H, and Figure 2, M and N). The supraoccipital crest serves as the attachment points of the epaxial muscles that generate force during suction feeding. These muscles are larger in Paxton benthic fish, contributing to a derived increase in suction index (McGee and Wainwright 2013). We hypothesized that these differences in supraoccipital crest morphology are adaptive for benthic feeding and have a heritable genetic component. We measured supraoccipital crest length, width, and area and a fourth skull trait, frontal width (interorbital distance). We identified 12 QTL that affect these skull traits (Table S4), including a large-effect QTL on chromosome 20 that significantly influenced supraoccipital crest width and area.

Opercle: The shape of the opercle bone (Figure 2O) also varies between marine and freshwater populations, which might reflect differences in feeding and/or respiration (Kimmel *et al.* 2005, 2012). Opercle size in this cross was strongly sexually dimorphic (Table S3) and was also controlled by seven autosomal QTL controlling opercle length, width, or neck width (Table S4).

Spines and median fins: We mapped QTL on 16 chromosomes controlling dorsal or anal spine length (Table S4). A QTL on chromosome 4 affecting the length of the second dorsal spine was the most significant QTL in our entire data set, with a LOD of 51. Chromosome 4 also had large effects on the lengths of the other two dorsal spines and the anal spine (Figure S7). In contrast, QTL on 7 chromosomes (2, 3, 5, 7, 8, 11, and 13) had regional effects with significant effects restricted to only one of the four spines measured. Chromosomes 3 and 2 had specific effects on dorsal spines 1 and 2, respectively, while chromosome 20 had regional effects on dorsal spine 3 and the anal spine (Figure 4). QTL controlling the number and area of serrations on the second dorsal spine (Figure 2P) mapped independently of the QTL controlling the length of the second spine, consistent with previous studies showing that presence or absence of serrations varies substantially among stickleback populations, even among populations with prominent second dorsal spines (Gross 1978).

Vertebrae traits: We found vertebrae number to be sexually dimorphic (Table S3), consistent with previous studies (Reimchen and Nelson 1987). Vertebrae number and position of axial landmarks were also under autosomal control, mapping to eight QTL. Although caudal vertebrae number mapped to one significant QTL, the caudal to abdominal vertebrae ratio, proposed to be important for larval fitness (Swain 1992b), had no detected QTL.

Covariance of traits and multivariate analysis of covariance

We analyzed patterns of trait covariance across all trait classes and found that in general, traits within a trait class tended to covary (Figure S8). As expected, traits that mapped strongly to the same chromosome (*e.g.*, gill raker and dorsal spine reduction, which both map strongly to chromosome 4) also tended to covary (Figure S8). The mean absolute correlations were 0.30 and 0.12 for traits within and between trait classes, respectively. We performed principal components analysis with all traits quantified in this study and mapped the first five principal components (Figure S9, Figure S10). The first principal component maps strongly to chromosomes 4 and 21 (Figure S9), and as expected, traits that map strongly to these two chromosomes (*e.g.*, branchial bone length, jaw size, and ventral pharyngeal tooth gain) load heavily onto this component (Figure S10). The second principal component maps strongly to chromosome 20 (Figure S9), and traits that map strongly to this chromosome (*e.g.*, gill raker reduction and supraoccipital crest shape) load heavily onto this component (Figure S10). Four of the top five principal components map significantly to chromosome 4 (Figure S9), suggesting that the patterns of trait covariance and integration are complex, but frequently involve particular stickleback chromosomes.

The genomic regions underlying trait clusters on chromosomes 4, 20, and 21 contain 48, 35, and 11 genes, respectively, that have Gene Ontology annotations suggesting key roles in early developmental patterning and signaling (see File S1). Changes in such genes may contribute to the multiple traits and covariances that map to particular stickleback chromosomes.

Most QTL are additive or partially additive

We estimated the dominance of each QTL using the formula d/a (Falconer 1989), where benthic alleles with strictly recessive, additive, and dominant effects have d/a values of -1, 0, and 1, respectively. Across all trait classes, there was a tendency for QTL to act additively, with the distribution of dominance values centered around 0 (Figure 5A). We defined dominance classes using d/a ranges as in a large sunflower domestication QTL study (Burke *et al.* 2002). Using these ranges, 28% of QTL are additive, 22% partially recessive, 21% partially dominant, 11% recessive, 5% dominant, 6% underdominant, and 6% overdominant (Table S6, Figure S11, Figure S12, Figure S13, Figure S14). QTL in different trait classes had similar patterns of dominance, with a consistent bias toward additive and partially recessive/dominant QTL (Table S6). We also observed an apparent relationship between effect size and dominance, with larger-effect QTL having more of an additive effect (Figure 5B, Figure S15), which simulations show is at least in part driven by a lower precision of dominance estimates of small-effect QTL (data not shown). To investigate possible bias in the detection of QTL differing in dominance, we carried out a simulation to determine the detection probability of additive and dominant QTL. Since more variance is present among the mean phenotypes of genotypic classes of a completely dominant or recessive QTL than an additive QTL, we predicted that additive QTL would be harder to detect than dominant QTL by interval mapping. As expected, the probability of detection was slightly higher for a dominant QTL than for an additive QTL (Figure S16). Thus, the detection of more QTL with additive effects does not result from a detection bias.

Although many different types of mutations can lead to loss or gain of structures during development, we tested the hypothesis that regressive or loss QTL (where the freshwater benthic allele contributes to smaller or fewer bones) might more often be recessive, while constructive or gain QTL (where the benthic allele contributes to larger or additional bones) might show more dominance. However, the sets of loss and gain QTL contained similar proportions of dominant QTL (5%), and the set of gain QTL actually showed a higher percentage of recessive QTL (16% *vs.* 7%, Table S6).

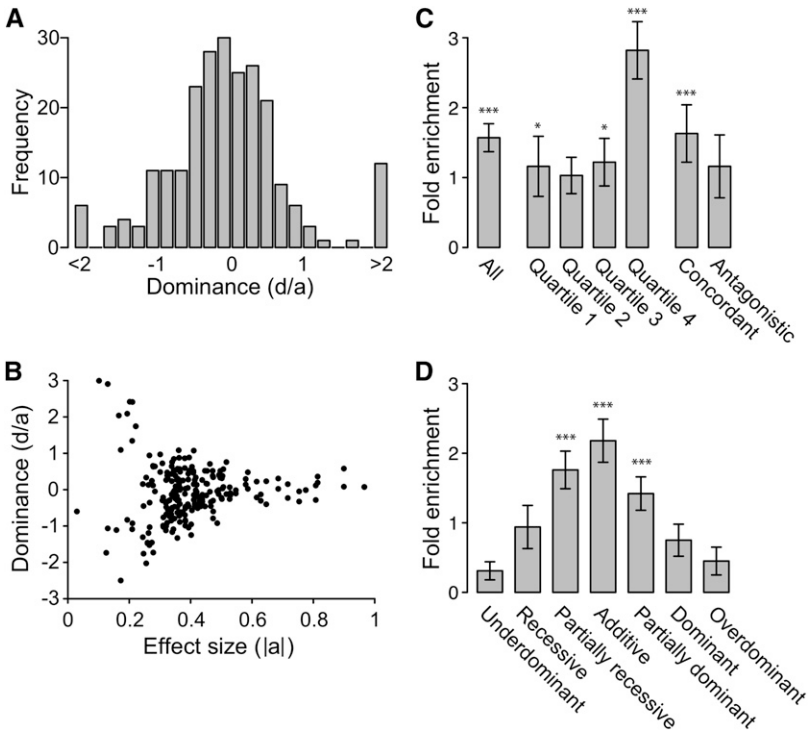

Figure 5 QTL dominance patterns and overlap with genomic intervals repeatedly selected during global marine–freshwater stickleback divergence. (A) Histogram of dominance values (*d*/*a*) of QTL reveals a tendency toward additive QTL (*d*/*a* = 0). (B) Dominance values for each QTL plotted against effect size (absolute value of *a*). Ten outlier dominance values that were either >3 or < −3 (File S3) are not shown. As effect size increases, QTL tend to be more additive. (C) Fold enrichment of QTL 1.5-LOD interval overlaps with genomic regions showing parallel marine–freshwater divergence in whole-genome sequence comparisons (Jones *et al.* 2012b) for all QTL, quartiles of QTL by LOD score, and concordant or antagonistic QTL. Highly significant enrichment is seen for all QTL, highest-LOD QTL (quartile 4), and concordant QTL. (D) Fold enrichment of QTL 1.5-LOD interval overlaps with signals of selection (Jones *et al.* 2012b) by dominance class. Highly significant enrichment is seen for partially recessive, additive, and partially dominant QTL, but not for other dominance classes. $*P < 0.05$, $***P < 0.001$.

For both sets of QTL, most loci were at least partially additive (69% for gain and 74% for loss, Table S6), and there was no significant difference ($P = 0.46$, Mann–Whitney *U*-test) between the distributions of dominances of loss and gain QTL.

QTL can also be classified into sets whose effects are either concordant or antagonistic to the overall direction of evolutionary change, (*i.e.*, where substitution of a benthic allele confers a more benthic-like or a more marine-like phenotype, respectively). Most (66%) QTL with a predicted evolutionary direction (based on known phenotypes from the grandparental populations) were in the concordant direction. Although we hypothesized that concordant and antagonistic QTL might show different dominance distributions, we observed no significant differences for the QTL identified in this study ($P = 0.61$, Mann–Whitney *U*-test).

Recent whole-genome resequencing studies in sticklebacks have identified a genome-wide set of regions that are consistently differentiated between marine and freshwater fish populations around the world and have likely been selected repeatedly to produce marine–freshwater differences (Jones *et al.* 2012b). The 1.5-LOD genetic intervals controlling skeletal traits in this QTL study were significantly enriched for the genomic regions that show consistent marine–freshwater sequence differences ($P < 0.001$, Figure 5C). The biggest enrichment was found for the genetic intervals that had the most significant effects on morphology, with a 2.8-fold enrichment ($P < 0.001$) observed for QTL in the top quartile of the LOD score (quartile 4, Figure 5C). Interestingly, we also observed significant enrichment for the set of concordant QTL that act in the same direction as overall evolutionary change (1.6-fold enriched, $P < 0.001$), but no significant enrichment for the set of antagonistic QTL (Figure 5C), as expected if marine–freshwater differentiated regions represent genomic intervals that are repeatedly selected to produce the consistent morphological differences observed in marine and freshwater environments. However, there was a trend toward enrichment for discordant QTL, and the difference in fold enrichment between concordant and antagonistic QTL was not significant ($P = 0.44$, two-tailed Student's *t*-test).

Some variants controlling freshwater stickleback phenotypes are carried at low frequency in marine populations (Colosimo *et al.* 2005; Miller *et al.* 2007), and the dominance of such variants may affect their carrier frequency in marine populations or the rate at which they increase in frequency following colonization of new freshwater environments. We therefore tested whether QTL in different dominance classes were differentially enriched for the marine–freshwater genomic regions that show evidence of repeated selection. Neither recessive nor dominant QTL were enriched for overlap with the genomic regions identified in the Jones *et al.* (2012b) study (Figure 5D). In contrast, partially recessive, additive, and partially dominant QTL were all strongly enriched for overlap with signals of repeated genomic selection ($P < 0.001$, Figure 5D).

Trait clusters on chromosomes 4, 20, and 21

As detailed above, inspection of QTL results revealed many complex and nonoverlapping patterns of genetic control within and among trait classes (Figure 3). However, certain chromosomes appeared enriched for QTL, especially QTL with high LOD scores, spanning multiple trait classes (Figure 3 and Table S4). To examine possible clustering in greater

detail, we first tested whether detected QTL were more likely to have peak markers within 5 cM of each other, compared to randomly distributed QTL (Protas *et al.* 2008). For both (1) all QTL and (2) large-effect QTL (defined as being in the top quartile by LOD), peak markers were significantly clustered ($P = 0.01$ and $P < 0.001$ for all QTL and large-effect QTL, respectively). In the entire set of filtered QTL, as expected, LOD score and PVE were highly correlated (Pearson's correlation coefficient = 0.95).

To ask whether specific chromosomes were significantly enriched for QTL, we used simulations to ask whether (1) all QTL or (2) large-effect QTL were overrepresented on any autosome. Relative to a null prediction where QTL are distributed in the genome in proportion to the genetic lengths of autosomes, large-effect QTL were significantly enriched on chromosomes 4, 20, and 21 (Figure 6). In contrast, only chromosome 21 was statistically enriched when we analyzed all QTL, not just those of large effect (Table S7). Since we statistically corrected for the effect of sexual dimorphism for each trait, we did not include the sex chromosome (chromosome 19) in these calculations.

Given that QTL are more likely to be detected in regions of low recombination ["the Noor effect" (Noor *et al.* 2001)], a low recombination rate on a particular chromosome could contribute to an enrichment of detected QTL on that chromosome. We therefore asked whether trait clustering was significant even when considering physical distance or gene number of each chromosome in the recently published stickleback genome assembly (Jones *et al.* 2012b). The enrichment of large-effect QTL on chromosomes 4 and 21 remains significant when compared to a null distribution of QTL generated in proportion to either chromosome length or gene number (Table S7), while the enrichment of QTL on chromosome 20 is suggestive, but not significant ($P = 0.14$ after correcting for either chromosome length or gene number).

For chromosomes 4 and 21, clustered traits included both loss and gain QTL, with benthic alleles in the same trait cluster contributing to bone loss for some traits and bone gain for others (Figure 7). For example, chromosome 4 had large effects on gill raker and dorsal spine loss, but also on jaw size gain. Chromosome 21 had large effects on tooth and branchial bone gain, but also on dorsal spine loss (Figure 7). In contrast, QTL mapping to chromosome 20 were in the direction where the benthic allele conferred loss or reduction of bone size across multiple trait classes.

Finally, we asked whether the three trait clusters on chromosomes 4, 20, and 21 are enriched for the genome-wide set of regions that are consistently differentiated between marine and freshwater stickleback populations (Jones *et al.* 2012b). These trait clusters on chromosomes 4, 20, and 21 overlapped 70, 26, and 6 marine–freshwater divergent regions from this set, respectively. Compared to the genome-wide average for an equivalently sized chromosome segment, the chromosome 4 and 20 trait clusters are significantly enriched ($P <$ 0.001 for both), with a 4.5-fold and 3.4-fold enrichment, respectively. The chromosome 21 trait cluster, in contrast, has only a 1.1-fold enrichment for these marine–freshwater divergent regions, which is not significant ($P = 0.28$). Thus, two of the three trait clusters are significantly associated with characteristic marine–freshwater divergent genomic regions.

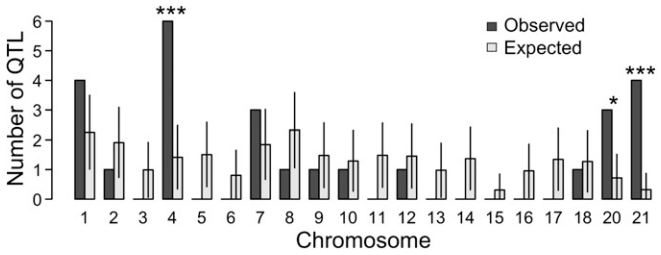

Figure 6 Large-effect QTL are enriched on chromosomes 4, 20, and 21. Observed (solid bars) and expected (open bars) numbers of large-effect QTL (top quartile by LOD) per chromosome are shown. Data for the expected values represent the mean and standard deviation of values generated from 10,000 simulations. This analysis excluded the sex chromosome (chromosome 19). $*P < 0.05$, $***P < 0.001$.

Discussion

Regional control of skeletal anatomy

A main finding of this study is that the genetic control of evolved skeletal morphology in sticklebacks involves both differential genetic control between trait classes and highly specific control of individual skeletal elements within a trait class. It is not surprising to find differential genetic control between trait classes, given the likely different embryonic origins of skeletal elements in different trait classes. Perhaps more surprising is the extent of highly specific anatomical control among skeletal elements thought to be serially homologous.

Consider, for example, the genetic mapping data for gill raker number. Gill rakers form throughout the pharynx, projecting from dorsal, joint, and ventral regions of branchial arches. The reduction in gill raker number in derived freshwater fish occurs in each branchial segment (Gross and Anderson 1984) and is typically described by summing all anterior-facing rakers on the first branchial segment [row 1 (*e.g.*, Hagen and Gilbertson 1972)]. Our mapping data revealed that in the Paxton benthic population, gill raker reduction was accomplished genetically in a piecemeal fashion by at least 23 QTL with specific effects in particular dorsal/ventral domains. Both of the large-effect gill raker QTL had regionally specific effects, the QTL on right chromosome 4 controlling anterior ventral gill raker number and spacing and the chromosome 20 raker QTL controlling strictly ventral gill raker number (Figure 4 and Figure S2). This decoupling of the genetic control of dorsal and ventral gill rakers is also consistent with a previous ecological study that found ventral, but not dorsal, gill raker number to have predictive value in discriminating different wild freshwater populations, perhaps reflecting population-specific diets (Reimchen *et al.* 1985). The anatomical specificity of QTL

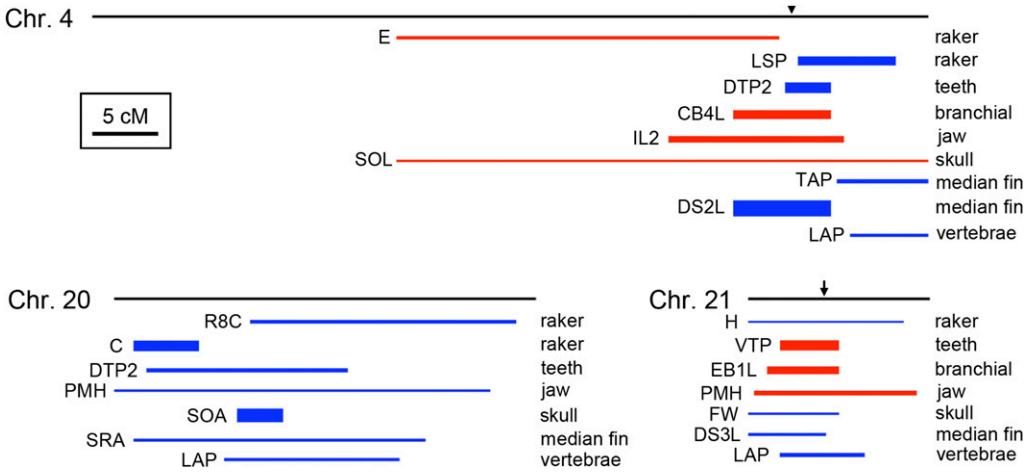

Figure 7 Trait clusters on chromosomes 4, 20, and 21. For each chromosome, the genetic length of the chromosome is shown as the black line at the top of each panel. (Bar, 5 cM.) Each blue, red, or gray bar represents a QTL, with the length of the bar denoting the 1.5-LOD interval. Trait abbreviations (Table S2) are listed to the right of each bar. The height of each bar is proportional to the LOD score of the QTL. For each QTL, direction of effect is indicated by red (benthic allele confers more or bigger bones) or blue (benthic allele confers fewer or smaller bones). The position of *Ectodysplasin* on chromosome 4 is marked with an arrowhead. A 1.7-Mb inversion on chromosome 21 (arrow), with different orientations typical in marine and freshwater fish (Jones *et al.* 2012b), mapped within the 1.5-LOD interval of all seven QTL on this chromosome, although the peak markers for each QTL (Table S4) mapped left of the inversion. See Figure 3, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7, and File S3 for more details on traits controlled by these three chromosomes. Abbreviations (see Figure 2 and Table S2): C, total cerato (ventral) gill raker number; CB4L, ceratobranchial 4 length; DS2L, dorsal spine 2 length; DS3L, dorsal spine 3 length; DTP2, dorsal toothplate 2 tooth number; E, epi (dorsal) gill raker number; EB1L, epibranchial 1 length; FW, frontal width; H, total hypo gill raker number; IL2, in-lever 2 of the articular; LAP, vertebrae number of last anal pterygiophore; LSP, lateral row 2 raker spacing; PMH, premaxilla height; R8C, row 8 cerato (ventral) gill raker number; SOL, supraoccipital crest length; SOA, supraoccipital crest area; SRA, spine 2 serration area; TAP, total postanal pterygiophore number; VTP, ventral toothplate tooth number.

might be even more complex; even within the ventral domain, the chromosome 20 raker QTL had regional effects, controlling only lateral and middle, but not medial spacing (Figure S2).

Another example of highly specific anatomical effects within a trait class is the genetic control of dorsal spine development. Nine of 14 QTL controlling dorsal spine lengths were specific to one of the three dorsal spines. Additional QTL controlled the number and area of barb-like serrations along the surface of the second dorsal spine, and these QTL mapped to different genomic regions than QTL that control length of the second dorsal spine. Previous studies have shown that natural populations of sticklebacks differ in the number of spines, the length of particular spines, and the degree of barb development along spines, likely reflecting the key roles of dorsal spine morphology in defense against different types of predators, as well as possible functions in display and dorsal pricking interactions during stickleback courtship (Hoogland *et al.* 1957; Gross 1978; Reimchen 1980; Kitano *et al.* 2009). Across larger phylogenetic distances, many other fish groups show striking changes in the length or morphology of individual spines, for example, the specific elongation of the first dorsal spine in trigger fish and angler fish. These dramatic species-specific modifications also likely depend on precise anatomical control of spine growth by genetic mechanisms that do not cause comparable changes in all members of a developmentally related series.

The high degree of regional control for skeletal QTL in sticklebacks is consistent with the idea that anatomically specific changes may avoid negative pleiotropy during development and will therefore predominate during morphological evolution in natural populations (Stern 2000; Carroll 2008). Highly specific skeletal effects may be controlled by genes whose expression patterns are themselves highly restricted along developmental axes (*e.g.*, *Hox* and *Dlx* genes) or by *cis*-regulatory changes that alter a particular subset of the expression domains of more broadly expressed genes. Further molecular dissection of the QTL mapped in this study, using genetic fine mapping and transgenic methods similar to those that have been successfully applied to other stickleback traits (Colosimo *et al.* 2005; Miller *et al.* 2007; Chan *et al.* 2010), should help illuminate the detailed mechanisms that vertebrates use to shape the size and number of individual skeletal elements as they evolve in different environments.

Most QTL had additive or partially additive effects

A second major conclusion from this study is that the majority of the detected evolutionary QTL had additive or partially additive effects, regardless of skeletal trait class and regardless of the overall direction of their effects on the skeleton (either gain or loss of bone in the derived freshwater form; Table S6). Our simulations indicated that detection bias does not explain the enrichment in additive QTL; instead it might contribute to an underdetection of additive QTL (Figure S16). The strong tendency toward additivity across trait classes suggests that this trend may be a general feature of evolved stickleback traits. Previous studies have shown that repressive genetic interactions tend to be as common as activating genetic mechanisms during development (Davidson and Levine 2008). Since constructive traits could be due to either loss of repressors or gain of activators, and regressive traits could arise by either loss of activators or gain of repressors, it is perhaps not surprising that a range of skeletal traits, including both increases and decreases of bony tissue, tend to show similar genetic architectures.

Two main models have been proposed for the effect of the dominance of a mutation on its likelihood of fixation during adaptation. "Haldane's sieve" predicts that new advantageous mutations are more likely to increase in frequency if they are not recessive (Haldane 1927). In contrast, Orr and Betancourt (2001) showed that if standing variants preexist in populations at mutation–selection balance and are disadvantageous prior to, but favored after, an environmental change, then probability of fixation in the new selective regime is largely independent of dominance. In the stickleback system, detailed case histories of the specific variants underlying armor and pigment traits (Colosimo *et al.* 2005; Miller *et al.* 2007) and more recent genome-wide surveys of parallel evolving freshwater populations (Hohenlohe *et al.* 2010; Jones *et al.* 2012b) show that repeated selection of ancient standing variants plays a substantial, but not exclusive (Chan *et al.* 2010), role in repeated marine–freshwater divergence. The overall distribution of dominance we observe for skeletal QTL in sticklebacks is thus likely based on a mixture of *de novo* mutations that have arisen during the divergence of the particular populations studied and older standing variants that likely exist at selection–migration balance in ocean populations, which become favorable when introduced into new freshwater environments. We observe a strong tendency toward additivity for QTL, which cannot be simply explained by either Haldane's sieve or the Orr/Betancourt model. However, the Haldane and Orr/Betancourt predictions are for fitness, and it is possible that the dominance for the skeletal traits studied here does not reflect the dominances for fitness, as previously seen in the dominances of *Eda* and chromosome 4 genotype on lateral plate morphology and fitness (Barrett *et al.* 2008).

Similar trends toward additivity of QTL have been observed in genetic studies of traits under artificial selection in mice and outbreeding plants (deVicente and Tanksley 1993; Burke *et al.* 2002; Kenney-Hunt *et al.* 2008; Ronfort and Glemin 2013), as well as in naturally evolved differences between surface and cave-adapted fish (Protas *et al.* 2008). This trend toward additivity for evolutionary QTL could result at least in part from a bias in the dominance distribution of the types of mutations favored by selection. For example, segregating *cis*-regulatory alleles have been found to be additive more often than *trans*-regulatory alleles (Lemos *et al.* 2008; McManus *et al.* 2010; Gruber *et al.* 2012). If selection favors *cis*-regulatory mutations, then additive QTL are expected to be common. Furthermore, the Orr/Betancourt model assumes that standing variation is at mutation–selection balance, whereas much of the standing variation reused by stickleback populations may be at migration–selection balance (Barrett and Schluter 2008), maintained in the ancestral marine population by introgression from freshwater populations. Such variation has already been filtered by selection: to be present in the sea it likely had increased in frequency already in freshwater populations. As a result, standing variation should be biased toward the kinds of mutations that selection favors in freshwater, which we hypothesize to be *cis*-regulatory mutations due to their low pleiotropy and a tendency to be additive.

Although we observed a strong tendency toward additivity of QTL, the 12% of overdominant and underdominant QTL observed likely indicates that some of the genetic effects observed in this cross result from complex interactions between the divergent grandparental genomes used for the cross, some of which may not be typical for very recent divergence between more closely related populations. Furthermore, although the extant Japanese Pacific marine population was used in this study as a living proxy for the marine ancestor of Pacific basin-derived freshwater fish including Paxton benthics, modern day marine fish cannot be equated with the ancestor of Paxton Lake fish. Given that genetic effects including dominance of QTL are likely context (*e.g.*, genetic background and environment) dependent, additional crosses will be needed to test general patterns of evolved genetic effects in this system.

Overall, the QTL identified in this study show significant enrichment for overlap with the previously identified haplotypes that are consistently differentiated between marine and freshwater fish populations around the world (Jones *et al.* 2012b). This enrichment suggests that a subset of the genomic regions repeatedly used for freshwater adaptation is selected for their effects on skeletal morphology. Large-effect QTL and additive QTL display the strongest enrichment, while small-effect, recessive, or dominant QTL show no or less enrichment for overlap with these haplotypes. These enrichment differences could at least partly result from a higher proportion of false positives in the set of small-effect QTL, which are more likely to be recessive or dominant. In addition, small-effect, recessive, and dominant classes might be enriched for new mutations (rather than standing genetic variation), which have a lower probability of detection by the method used in the Jones *et al.* (2012b) study. Further analysis of the QTL intervals identified in this study will test the hypothesis that the enrichment of these signals of selection in the QTL intervals is driven by particular genomic regions that act to control specific skeletal traits mapped in this study. Future population genetic studies in marine and Paxton benthic populations can also test whether haplotypes inside the QTL intervals identified here are outliers for metrics such as F_{st}.

Clustering of QTL on chromosomes 4, 20, and 21

A third major finding of this study is that multiple trophic and armor traits map strongly to chromosomes 4, 20, and 21. We found that QTL from six (chromosome 20) or seven (chromosomes 4 and 21) of the eight trait classes mapped to each trait cluster (Figure 3). Although all QTL were enriched only on chromosome 21, large-effect (top quartile of QTL by LOD) QTL were enriched on chromosomes 4, 20, and 21 (Figure 6). All three trait clusters controlled specific subsets of skeletal traits and are thus unlikely to represent loci generally involved in bone formation. For example,

some skeletal traits, such as opercle size, mapped strongly to multiple genomic locations but were not significantly controlled by any of the three large-effect trait clusters.

The trait clusters could result from single genes with pleiotropic effects or from the combined effects of multiple linked genes. Several QTL studies have identified loci that are thought to have pleiotropic consequences (Kimura *et al.* 2007; Albert *et al.* 2008; Studer and Doebley 2011), including a large-scale study of QTL controlling skeletal differences between mice artificially selected for large or small body size (Kenney-Hunt *et al.* 2008). In contrast, genetic studies in butterflies, pinthrum, and *Petunia* have reported some trait clusters that are due to closely linked but separable loci, rather than to pleiotropic effects of a single gene (Kurian and Richards 1997; Joron *et al.* 2006; Ferguson *et al.* 2010; Hermann *et al.* 2013). As the degree of pleiotropy of a QTL increases, the relative frequency of antagonistic effects (effect in the opposite direction of the direction of evolutionary change) is predicted to increase during selection (Griswold and Whitlock 2003), which perhaps at least partially explains our observation that a significant fraction (34%) of QTL are antagonistic. However, antagonistic effects could also result from stabilizing selection, from genetic drift (Rieseberg *et al.* 2002; Griswold and Whitlock 2003), or from pleiotropic mutations that overshoot the optimum phenotype.

For two of the stickleback trait clusters presented here, genetic resolution of linked traits argues against pleiotropy. For example, two of the linked raker QTL on chromosome 4, as well as two linked raker and supraoccipital crest QTL on chromosome 20, appear spatially distinct from each other, with nonoverlapping 1.5-LOD intervals (Figure 7). In addition, for the trait clusters on chromosomes 4 and 21, benthic alleles do not act in a consistent phenotypic direction (Figure 7). For example, chromosome 4 benthic alleles reduce gill raker number, pharyngeal tooth number, and dorsal and anal spine lengths, but also increase upper and lower jaw sizes, branchial bone sizes, and the length of the supraoccipital crest. Given the opposite directions of phenotypic effects and the genetic resolution separating some of the linked QTL, we favor a model where several individual, linked QTL exist, possibly including a supergene complex with multiple effects on both armor and trophic phenotypes. Increased genetic resolution of these overlapping QTL is needed to test whether the QTL are separable and whether some of the overlapping traits might resolve to a supergene complex. In cases where loss and gain QTL overlap, it is possible these traits share developmental interactions (*e.g.*, the genetically encoded loss of a trait might result indirectly in the gain of another).

In cases of multiple linked QTL, trait clustering may be due to genomic intervals of decreased recombination. For example, inversions suppress recombination and in *Mimulus* and *Heliconius* appear to lock in a suite of coadaptive polymorphisms (Lowry and Willis 2010; Joron *et al.* 2011; Fishman *et al.* 2013). Recent stickleback genome sequencing revealed a 1.7-Mb inversion on chromosome 21 that displays strong signals of selection, whereby marine and freshwater populations have high and low allele frequencies, respectively, of the inversion (Jones *et al.* 2012b). Jones *et al.* (2012b) proposed that this inversion may hold several distinct adaptive loci together, and both the current study of skeletal QTL and another recent study of lateral line QTL (Wark *et al.* 2012) confirm that many QTL map to chromosome 21, with confidence intervals that overlap the position of the inversion. Although this study identifies a large number of new traits that may be controlled by an inversion/supergene complex in sticklebacks, we note that the peak markers for each of the chromosome 21 QTL map left of the inversion. Ongoing fine-mapping studies using crosses that generate recombination events in and around the inversion will provide useful information on both the position and the identity of the genes and mutations that underlie one of the most distinctive trait clusters in the stickleback genome.

Previous studies have identified multiple QTL mapping to chromosome 4 in sticklebacks, including QTL for lateral plate number and lateral plate size (Colosimo *et al.* 2004; Cresko *et al.* 2004), pelvic spine length (Shapiro *et al.* 2004), and multiple aspects of body shape (Albert *et al.* 2008; Rogers *et al.* 2012). The data presented here reveal that a surprisingly large number of additional traits also map to chromosome 4, including gill raker number, pharyngeal tooth number, branchial bone size, premaxilla size, dentary and articular size, supraoccipital crest length, dorsal and anal spine length, and aspects of vertebral positioning. Many of these traits, including larger jaws and fewer gill rakers, shorter dorsal and pelvic spine lengths, reductions in lateral plate number, and changes in overall body shape, appear to have adaptive significance in benthic environments, as multiple benthic species independently evolve these morphological changes in recurrent stickleback species pairs (Schluter and McPhail 1992). Linkage of large-effect QTL controlling multiple aspects of both trophic morphology and antipredator defense may preserve combinations of traits that function together in different ecological environments. For example, fish foraging in open water environments not only specialize on different food sources, but also tend to encounter different predators. Thus, tight linkage of genes controlling feeding and armor traits may provide a fitness advantage to offspring of contrasting ecotypes, and theory predicts that such linked assemblages will evolve under conditions where strongly contrasting forms sometimes meet and hybridize (Kirkpatrick and Barton 2006), as frequently occurs in marine–stream and benthic–limnetic stickleback species pairs.

Parallel evolution of polygenic traits

Large-effect QTL for armor plate, pigment, and pelvic development that were previously mapped in this cross do not appear to be specific to this cross. Instead the same major loci (Colosimo *et al.* 2004; Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007), the same underlying genes

(Chan *et al.* 2010), and sometimes even the same freshwater alleles (Colosimo *et al.* 2005; Miller *et al.* 2007) are used repeatedly in other populations that have evolved similar phenotypes (Jones *et al.* 2012a). All of these well-studied examples involve QTL that control half or more of the variance in the corresponding trait, and it remains unclear whether QTL with smaller effects, like many of those identified here, will also be used in parallel in other populations. Previous studies have mapped two gill raker number and four dorsal spine length QTL in a Priest Lake benthic × limnetic cross (Peichel *et al.* 2001). Both of the Priest Lake raker QTL overlap raker QTL found in this study, although anatomical domains affected by these QTL differ. In contrast, only one of the four Priest Lake dorsal spine QTL overlaps any of the spine QTL presented here. Although the Priest Lake cross also used benthic forms, it was a backcross to freshwater limnetic fish, and trophic and armor selective pressures likely differ on limnetic *vs.* marine fish. It is also likely that some genetic variation is not fixed within a population and that the spectrum of QTL observed in a genetic cross could be different if different individuals from the same population were used. Additional crosses are needed to test whether similar genetic loci underlie repeated evolution of similar trophic and armor phenotypes in many benthic lake and stream forms that have evolved from marine ancestors.

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