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Partially repeatable genetic basis of benthic adaptation in threespine sticklebacks

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The extent to which convergent adaptation to similar ecological niches occurs by a predictable genetic basis remains a fundamental question in biology. Threespine stickleback fish have undergone an adaptive radiation in which ancestral oceanic populations repeatedly colonized and adapted to freshwater habitats. In multiple lakes in British Columbia, two different freshwater ecotypes have evolved: a deep-bodied benthic form adapted to forage near the lake substrate, and a narrow-bodied limnetic form adapted to forage in open water. Here, we use genome-wide linkage mapping in marine × benthic F2 genetic crosses to test the extent of shared genomic regions underlying benthic adaptation in three benthic populations. We identify at least 100 Quantitative Trait Loci (QTL) harboring genes influencing skeletal morphology. The majority of QTL (57%) are unique to one cross. However, four genomic regions affecting eight craniofacial and armor phenotypes are found in all three benthic populations. We find that QTL are clustered in the genome and overlapping QTL regions are enriched for genomic signatures of natural selection. These findings suggest that benthic adaptation has occurred via both parallel and nonparallel genetic changes.

KEY WORDS: **Convergent evolution, genotyping-by-sequencing, parallel evolution, QTL, skeleton.**

Convergent evolution, the evolution of similar phenotypes in independent lineages adapting to similar ecological niches, provides compelling evidence for ecological adaptation (Schluter 2000), as well as natural replicates to study the genetic basis of evolutionary change. Convergent phenotypic evolution sometimes occurs through changes in the same genes in multiple lineages, called parallel evolution (Stern 2013), suggesting some evolutionary trajectories are constrained and partially predictable. How often convergent evolution occurs through parallel and thus predictable genetic changes remains an outstanding and important question (Stern and Orgogozo 2008, 2009; Rosenblum et al. 2014). While many studies have identified similar genetic bases for convergent phenotypes (reviewed in Conte et al. 2012; Stern 2013), few studies have simultaneously tested for genetic parallelism underlying many phenotypic traits in multiple convergently evolved populations.

All data are available in the Supplemental Data File.

One critical first step in testing for a parallel genetic basis of convergence is mapping the genomic regions involved in convergent phenotypic evolution. Although quantitative trait locus (QTL) mapping can robustly identify genomic regions underlying complex traits, the genomic intervals are typically large and contain multiple genes when there are few rounds of recombination in the genetic cross. However, comparing the genomic intervals of QTL between crosses from different populations will either support or refute the hypothesis of genetic parallelism, since QTL can either map to overlapping regions (potentially parallel) or map to nonoverlapping regions (nonparallel).

Threespine stickleback fish (*Gasterosteus aculeatus*) provide a powerful vertebrate model system to study convergent phenotypic evolution, as ancestral oceanic forms have repeatedly colonized and rapidly adapted to countless freshwater environments throughout the Northern hemisphere (Bell and Foster 1994). Many morphological phenotypes convergently

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evolve in freshwater, most of which are likely adaptations to the new ecological pressures in freshwater environments, such as a different diet and predation regime. In five drainages in British Columbia, two freshwater species pairs convergently evolved: a benthic species adapted to feed on the lake bottom, and a limnetic species adapted to forage in the open water (McPhail 1984, 1992; Schluter and McPhail 1992). Across lakes, the different benthic and limnetic forms strikingly resemble each other, yet evolved in isolation (Taylor and McPhail 1999). Once thought to be the result of sympatric speciation, phylogenetic analyses based on allozymes (McPhail 1984, 1992), nuclear microsatellites (Taylor and McPhail 2000), mtDNA haplotypes (Taylor and McPhail 1999), genome-wide SNP genotypes (Jones et al. 2012a), and salinity tolerance experiments (Kassen et al. 1995) instead support a double invasion scenario. In this scenario, the first oceanic colonization event evolved into a freshwater form, and then a second oceanic colonization event displaced the first population to the benthic niche while adapting to the alternative open water limnetic niche (Taylor and McPhail 2000). Thus, the distinct benthic morphs found in species pair lakes are especially divergent from marine ancestors and offer an outstanding system to study the genetic architecture of repeatedly but independently evolved phenotypes.

Differences in a number of skeletal traits have evolved repeatedly in benthic sticklebacks. Benthic fish have fewer gill rakers, a larger jaw, and reduced pelvic and dorsal spines (McPhail 1984, 1992, 1994; Schluter and McPhail 1992). Additionally, benthic fish from at least one of these species pair lakes, Paxton Lake, have evolved more pharyngeal teeth and longer branchial bones relative to marine fish (Cleves et al. 2014; Erickson et al. 2014). Collectively, these craniofacial differences have important functional consequences: benthic fish are more efficient at foraging for larger prey items in a benthic substrate, and limnetic fish are more efficient at foraging for plankton from open water (Bentzen and McPhail 1984; Schluter 1993). In turn, the distinct morphologies are important for survival in their respective niches; benthic-limnetic hybrids have reduced survival in nature (Gow et al. 2007). Some of these benthic trophic traits have also been documented in nearby lakes that have a predominantly benthic environment yet only have a single ecomorph (Lavin and Mcphail 1985), suggesting that many of these traits are important for adaptation to a benthic environment, either with or without a limnetic ecomorph.

How often do the same genomic regions underlie convergent phenotypic adaptation in multiple benthic environments? Hybridization occurs between anadromous marine and freshwater sticklebacks (Hagen 1967; Jones et al. 2006, 2008), and likely maintains freshwater-adapted alleles at low frequency in oceanic populations (Barrett and Schluter 2008; Schluter and Conte 2009; Hohenlohe et al. 2012; Jones et al. 2012b; Terekhanova et al. 2014), which might increase the chances of parallel evolution. For example, an ancient allele of *Eda* conferring reduced lateral plate armor is present at low frequency in marine populations and has been reused many times in freshwater adaptation (Colosimo et al. 2004, 2005; O'Brown et al. 2015). Allele sharing by hybridization of differently adapted populations has also been found to be an important contributor to parallel evolution in other species, including *Heliconius* butterflies, Galapagos finches, mice, humans, and *Mimulus* (The Heliconius Genome Consortium 2012; Lamichhaney et al. 2015; Song et al. 2011; Huerta-Sánchez et al. 2014; Stankowski and Streisfeld 2015). However, convergent evolution by novel mutations in the same gene or genetic pathway has also been observed in sticklebacks and other systems (Colosimo et al. 2004; Protas et al. 2006; Kingsley et al. 2009; Chan et al. 2010; Rosenblum et al. 2010; Vickrey et al. 2015). Alternatively, convergent phenotypic evolution may have a mostly different (nonparallel) genetic basis (Wittkopp et al. 2003; Kowalko et al. 2013; Ellis et al. 2015; Glazer et al. 2015). The overall extent to which parallel versus nonparallel genetic changes and new mutations versus standing variation are involved in stickleback evolution remains largely unknown (Conte et al. 2015).

Here, we use genome-wide linkage mapping to map QTL influencing a variety of trophic and armor traits in three marine $\times$ benthic F2 genetic crosses, each using a benthic grandmother from independently derived benthic populations in Paxton, Priest, and Enos Lakes. The shared marine grandfather individual came from an anadromous marine population geographically near the benthic populations (Little Campbell River, BC), and thus serves as an extant proxy for the marine population that likely colonized the lakes. This crossing scheme allows identification of genomic regions responsible for phenotypic differentiation of each benthic population compared to a shared marine genetic background. In the three crosses, QTL that map to overlapping genomic regions and influence similar phenotypes in multiple crosses are candidates for parallel genetic evolution. Alternatively, and assuming no major chromosomal rearrangements, QTL mapping to unique genomic regions indicate nonparallel evolution.

Methods

ANIMAL STATEMENT AND CROSSES

A single wild Little Campbell marine (LCM, Little Campbell River, British Columbia) male was crossed to wild benthic freshwater females from Paxton Lake (PAXB, Texada Island, BC), Priest Lake (PRIB, Texada Island, BC), and Enos Lake (ENOB, Vancouver Island, BC) in 2002. See Figure 1A for representative fish from each population and maps of population locations (Fig. 1B and C). Since the Enos species pair collapsed between 1994 and 2002 (Kraak et al. 2001; Taylor et al. 2006), a female from Enos lake that morphologically resembled the benthic ecomorph was used. Fish were raised in 100 l aquaria in 5 ppt

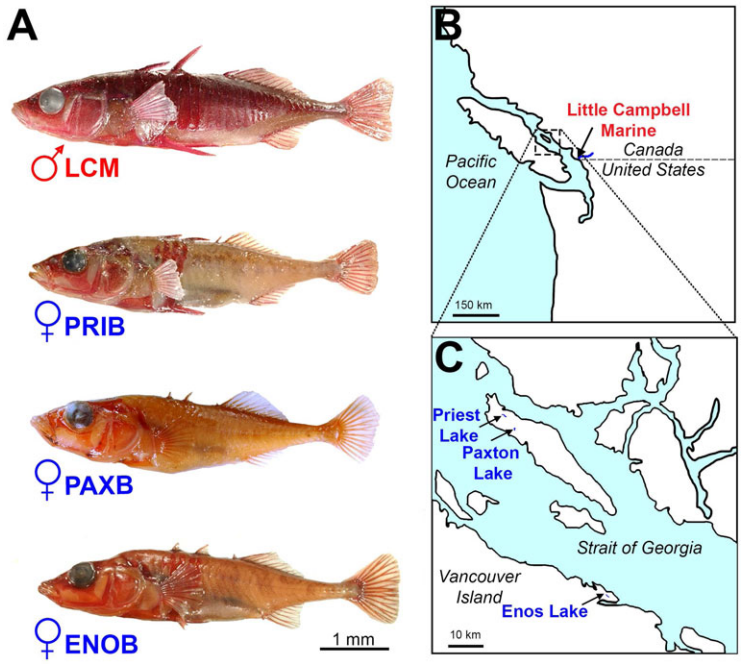

Figure 1. **Benthic populations studied. (A) Representative wild-caught adult fish from each population, with bone stained with Alizarin red. LCM = Little Campbell Marine, PAXB = Paxton Benthic, PRIB = Priest Benthic, ENOB = Enos Benthic. (B, C) Location of the marine population source and three species-pair lakes in the Pacific Northwest. The Little Campbell River, where the anadromous LCM population breeds, is indicated with an arrow in (B).**

saltwater. F1 fish were intercrossed to produce a total of five F2 families (see Table S1). One family of 180 F2s was studied for the ENOB cross; 186 F2s (two families of 94 and 92) for the PAXB cross, and 180 F2s (two families of 90) for the PRIB cross. F2s were raised to six months, euthanized, and stored in ethanol. All animal work was approved by the UBC Animal Care committee under protocol A00-191.

GENOTYPING, PHENOTYPING, LINKAGE MAP CONSTRUCTION, AND QTL MAPPING

Thirty-six skeletal phenotypes in ten trait categories (Fig. S1, Table S2) were measured in F2s as previously described (Miller et al. 2014) and corrected for standard length and sex when appropriate. DNA was extracted from pectoral fins, F2s genotyped using Genotyping by Sequencing (GBS) in two Illumina HiSeq 2000 lanes using 384 barcoded samples per lane, and sequence reads analyzed using a custom pipeline based on SNPs with opposite homozygous genotypes in the grandparents as described in Glazer et al. 2015 (Table S3). Linkage maps based on 805 binned markers shared in all three crosses were constructed in JoinMap 4.0 (Kyazma). The *stepwiseqtl*, *addqtl*, *refineqtl*, and *fitqtl* functions of R/qtl were used to identify QTL significant at a genome-wide significance level of LOD = 3.7. See Supporting Online Material for additional genotyping, phenotyping, and QTL mapping methods.

For analysis of phenotypic variation at specific markers, the *argmax.geno* function of R/qtl was used to calculate the most likely genotype for fish with missing genotypes at that marker. Dominance and additivity of each QTL were calculated based on the phenotypic means for each genotypic class at the peak marker (Falconer and Mackay 1996). In the PAXB cross only, a subset of F2s lacking pelvic spines were excluded from the analysis of pelvic spine length. In this cross, pelvic spine presence/absence was mapped separately as a binary trait with the *scanone* function.

IDENTIFYING SUGGESTIVE PARALLEL QTL

The minimum QTL effect size that can be detected is larger when cross size is smaller (Beavis 1998), so detecting small effect QTL shared in multiple relatively small crosses is even less likely. To minimize the resulting bias against detecting parallelism, especially given the limitations of the cross sizes of ~180 F2s per each of three crosses studied here, we considered QTL identified in the genome-wide search to be "candidate" QTL for each trait class (as in Conte et al. 2015). If no QTL for the same trait class were found on the same chromosome on which the candidate QTL was detected in a second cross, we then looked for suggestive parallel QTL that overlapped the candidate QTL as follows. We tested for QTL having LOD scores above 2.0 on the same chromosome for all phenotypes in the same trait class in the

second cross. We then added these QTL to the *refineqtl* model to recalculate the LOD scores and 1.5 LOD intervals for the QTL model. If the peak marker of the suggestive QTL was found within the 1.5 LOD interval of any candidate QTL in the same trait class from either of the other two crosses, we considered the QTL to be a suggestive parallel QTL and included it in the supplemental analysis of suggestive QTL. QTL that did not meet this criterion were removed. All analyses involving suggestive parallel QTL used the QTL locations and percentage of phenotypic variance explained (PVEs) estimated by the *refineqtl* model that included suggestive parallel QTL.

QTL FILTERING

To minimize overcounting QTL for multiple similar phenotypes within a trait class, we generated a list of filtered QTL as follows. For each of the seven trait classes with multiple phenotypes (teeth, gill rakers, branchial bones, median fin, pelvic spines, jaw, and opercle), we identified the largest effect QTL (highest PVE) on each chromosome for that trait category in each cross. Since many of the phenotypes measured are serially repeated traits (such as gill raker count or ceratobranchial length), this filtering method avoids overrepresentation of QTL influencing multiple anatomically similar traits, following a previous analysis of many stickleback skeletal QTL (Miller et al. 2014). The filtering approach was applied to both genome-wide and suggestive parallel QTL. Hereafter, unless otherwise specified, QTL refers to these filtered QTL (Tables S4 and S5).

QTL CLUSTERING

We tested for clustering of QTL as described (Arnegard et al. 2014). Given the total number of QTL detected in each cross, we calculated the expected number of QTL per chromosome based on genetic length, physical length, and number of Ensembl gene predictions per chromosome. Physical length and gene number were based on the revised assembly of Glazer et al. 2015 and genetic length was based on the linkage map for each cross. We then used the R function *chisq.test* to test whether the observed number of QTL per chromosome differed significantly from the expected number. *P*-values were calculated based on 10,000 permutations of the data. In crosses in which the distribution differed significantly from the expected number ($P < 0.05$), we used the standard residuals from the chi-square test to determine which chromosomes were enriched for QTL, with a standard residual >2 considered enriched.

OVERLAP ANALYSIS

We determined overlap by asking whether the physical 1.5 LOD intervals of QTL for the same trait category overlapped between crosses. Unique QTL were identified if they did not overlap with a QTL found in any other cross. Double overlaps were identified if they overlapped in only two of the three crosses. Triple overlaps were identified if the 1.5 LOD intervals overlapped a region of the genome in all three crosses. We calculated the mean PVE of each double and triple overlap by averaging the individual PVEs of the overlapping QTL. To test whether the QTL datasets were enriched for overlapping QTL, we randomly permuted the physical locations of the QTL in the genome 10,000 times. In each permutation, no two QTL in the same trait category in the same cross could have overlapping 1.5 LOD intervals. We then calculated the number of unique QTL, double overlaps, and triple overlaps for each permutation. We compared the actual number of overlaps between crosses to the distribution of simulated number of overlaps and calculated a *P*-value for the extent of overlap based on the percentage of permutations in which the number of overlaps was equal to or greater than the observed number.

ANALYSIS OF SIGNALS OF SELECTION

Two previous studies identified regions of the stickleback genome that show strong signatures of natural selection in 21 populations (Jones et al. 2012b) or in benthic populations relative to their limnetic counterparts (Jones et al. 2012a). As in the overlap analysis, we tested for an enrichment of these signals of selection in the total set of QTL found here by randomly permuting the locations of the 1.5 LOD intervals of the QTL 10,000 times for each cross. We then compared the number of signals of selection overlapping QTL in the permuted dataset to the number of actual overlaps with the signals of selection. We calculated fold enrichment for each cross based on the ratio of the actual number of overlaps relative to the permuted mean. The *P*-value was calculated as the percent of all permutations in which the number of overlaps was greater than or equal to the observed number of overlaps. For overlapping double or triple QTL, the maximum physical range spanned by the 1.5 LOD intervals of all two or three QTL was used in calculations.

For the genomic regions displaying marine-freshwater signals of selection in 21 stickleback genome sequences (Jones et al. 2012b), we used the union of the HMM and CSS signals of selection (a total of 240 regions) and calculated their locations in the revised genome assembly from Glazer et al. 2015. Each time the 1.5 LOD interval of a QTL overlapped with a signal of selection was counted as an overlap. For the benthic-limnetic signals of selection, we used all 46 F_{ST}-outlier SNPs identified in any one of the three species-pair lakes (Paxton Lake, Priest Lake, or Quarry Lake; Jones et al. 2012a) and converted the SNP location to the revised genome assembly (Glazer et al. 2015). We then counted every overlap between a QTL and an F_{ST}-outlier SNP and compared this number to the simulated number of overlaps.

Results

OVERLAPPING REGIONS OF THE GENOME AFFECT ARMOR AND CRANIOFACIAL TRAITS IN MULTIPLE BENTHIC POPULATIONS

To test whether similar genetic architectures underlie skeletal adaptation in multiple populations of benthic sticklebacks, we phenotyped 36 skeletal traits in ten different trait categories in three marine × benthic crosses (see Table S2) and found a strong correlation between phenotypes within each trait category within each cross (see Fig. S1). We used genotyping-by-sequencing (GBS, Elshire et al. 2011; Glazer et al. 2015) to generate genome-wide genotypes with at least a 50% success rate in 546/554 (98.5%) of F2s sequenced (see Table S3). Using random permutations of 22 phenotypes, we calculated a genome-wide significance threshold of LOD 3.7 for QTL mapping. All traits except standard length (SL), basihyal length (BH), and premaxilla length (PML) mapped significantly to at least one chromosome in at least one cross with this cutoff. Our linkage maps were all collinear with the revised genome assembly (Glazer et al. 2015), suggesting no major genome rearrangements occurred in the parents of the crosses.

We identified a total of 157 QTL (46 PAXB, 64 PRIB, and 47 ENOB) significantly affecting skeletal traits in the three crosses (see Fig. 2A, and Table S4). We then filtered the QTL from each cross such that only the QTL with the highest percentage of phenotypic variance explained (PVE) in a trait class was kept for each chromosome to minimize redundant oversampling of QTL (following Miller et al. 2014). This filtering resulted in a total of 100 QTL (33 PAXB, 40 PRIB, and 27 ENOB). Overall, the effect sizes of filtered and unfiltered QTL were quite similar: most QTL were small effect (PVE < 20), with a few QTL of large effect (see Fig. S2 for the distribution of PVE of all QTL and all filtered QTL in each cross). QTL overlapped if the physical ranges of the 1.5 LOD intervals overlapped in two or three crosses. We observed that 43% of all filtered QTL overlapped with a QTL influencing a trait in the same category in at least one other cross.

We found six overlapping QTL between PAXB and PRIB, two between PAXB and ENOB, and two between PRIB and ENOB (Fig. 2B). Ninety percent (9/10) of all QTL overlapping in two crosses had effects in the same direction. Eight QTL overlapped in all three crosses (Figs. 2B, 3, and 4). Five of these QTL affected armor traits and were found on chromosome 4 (dorsal spine length, pelvic spine length, and lateral plate number, Fig. 3A–C), chromosome 21 (pelvic spine, Fig. 3D), and chromosome 7 (lateral plates, Fig. 3E). Notably, the chromosome 4 QTL affecting dorsal and pelvic spines (Fig. 3A and B) had large effects in all three crosses and mapped to a similar region of chromosome 4 in all three crosses. However, the genetic basis of pelvic and dorsal spine length are markedly different: several QTL were identified that affect pelvic but not dorsal spine length (Fig. 2). For all triple-overlapping armor QTL, the benthic allele conferred a reduction in the number or size of skeletal element measured, except for the chromosome 7 lateral plate QTL in PRIB, for which heterozygotes had the fewest plates (Table S4).

The genetic and developmental bases of several QTL influencing freshwater trophic adaptation in sticklebacks have been studied extensively (Cleves et al. 2014; Erickson et al. 2014; Glazer et al. 2014; Ellis et al. 2015). We were particularly interested in whether previously identified QTL affecting the branchial skeleton (the major food-processing apparatus in fish) were found in multiple benthic populations, which could suggest that these QTL are under selection in benthic environments. A large-effect QTL increasing ventral pharyngeal tooth gain on chromosome 21 (Miller et al. 2014; Cleves et al. 2014) was indeed found in all three crosses (Fig. 4A), with the benthic allele conferring more teeth. However, the PRIB cross appeared to have two QTL peaks on chromosome 21 (Fig. 4A). We found that a gill raker reduction QTL on chromosome 4, previously described to overlap in three independently derived freshwater populations (including PAXB, Glazer et al. 2014), was also found in all three benthic populations, as judged by overlapping 1.5 LOD intervals (Fig. 4B). However, a second previously identified QTL on chromosome 20 was found only in the PAXB and PRIB crosses (Table S8). Previously described QTL for increased branchial bone length (Erickson et al. 2014) on chromosomes 4 and 21 were each found in two crosses but were found in all three crosses when suggestive parallel QTL were included (Fig. 4C–D). However, the suggestive ENOB chromosome 21 QTL appeared to map to a different region of the chromosome (although it was counted as an overlap because the peak marker was found within the 1.5 LOD interval of the PRIB CB5 QTL, which was in the same branchial bone trait category). We also found a previously unreported QTL on chromosome 8 that influenced opercle width in all three crosses (Fig. 4E).

BENTHIC QTL FOR SIMILAR TRAITS OVERLAP MORE THAN EXPECTED BY RANDOM CHANCE

We hypothesized that abundant standing genetic variation (Colosimo et al. 2005; Conte et al. 2012; Bell and Aguirre 2013) and similar selective pressures in the benthic freshwater environments would lead to more genetic parallelism in QTL affecting skeletal traits than expected by chance. To test for significant parallelism, we randomly permuted the locations of the QTL in the genome without replacement for each trait category and calculated the number of overlapping QTL in each permutation (Fig. 2B, see Fig. S3 for the distributions of simulated overlapping QTL). We found that the observed number of triple-overlapping QTL significantly exceeded the number of triple QTL expected by chance (eight observed triple overlaps vs. a maximum of five

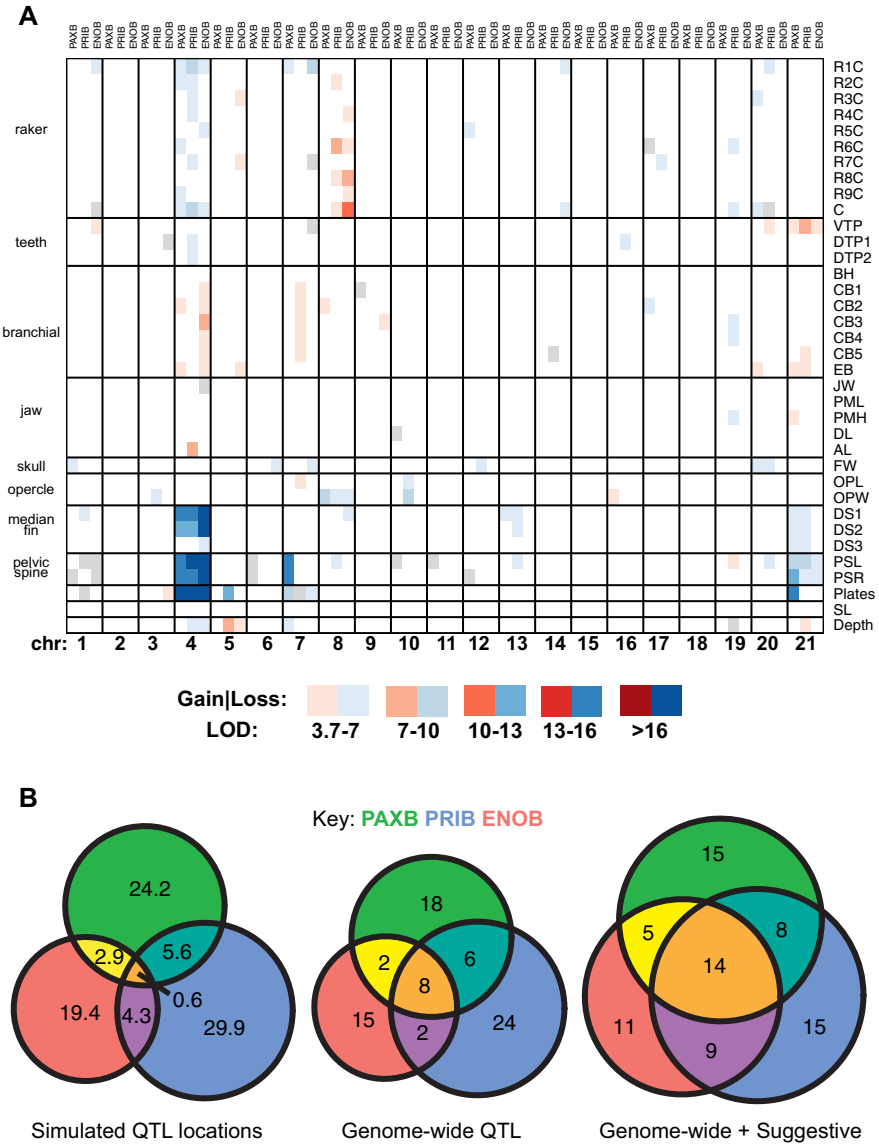

Figure 2. **Overview of QTL. (A) Summary of QTL detected at a LOD 3.7 genome-wide significance threshold. Chromosomes 1–21 are separated by vertical lines and numbered below. Within each chromosome, QTL for PAXB, PRIB, and ENOB (labeled on top) are indicated from left to right. Trait categories are labeled at left and separated by horizontal lines; descriptions of phenotype abbreviations at right can be found in Table S2. Color intensity indicates magnitude of LOD score (see key). Red colors indicate skeletal gain QTL (freshwater allele confers more bone); blue colors indicate skeletal loss QTL (freshwater allele confers less bone). QTL in which the phenotypes of homozygous genotypes do not differ by a Student's *t*-test ($P > 0.05$) are shaded in gray. (B) Venn diagrams of simulated QTL overlap (left), all genome-wide QTL overlap (middle), and all QTL including suggestive parallel QTL (right).**

in 10,000 permutations of the data, Table 1). However, double QTL were not significantly enriched in any population pair. All three crosses had significantly fewer unique QTL than expected by chance at a $P < 0.05$ significance level (98–99% of permutations had equal or more unique QTL, respectively, Table 1). Combined, these results suggest that strong selection on some skeletal traits may drive genetic parallelism for the QTL that were found to overlap in all three lakes, which then may result in a concomitant dearth of double and unique QTL. Despite this finding, the majority of detected QTL (57/100 QTL) were unique to a single cross, suggesting that benthic adaptation also has a large nonparallel component.

The number of overlapping QTL might be overcounted if mutations are present that affect the development of skeletal elements belonging to more than one trait class. In total, four distinct genomic regions contain triple QTL, which is still significant in a modified permutation test that disallows multiple triple QTL in the same genomic region ($P = 0.001$). Therefore, we still find

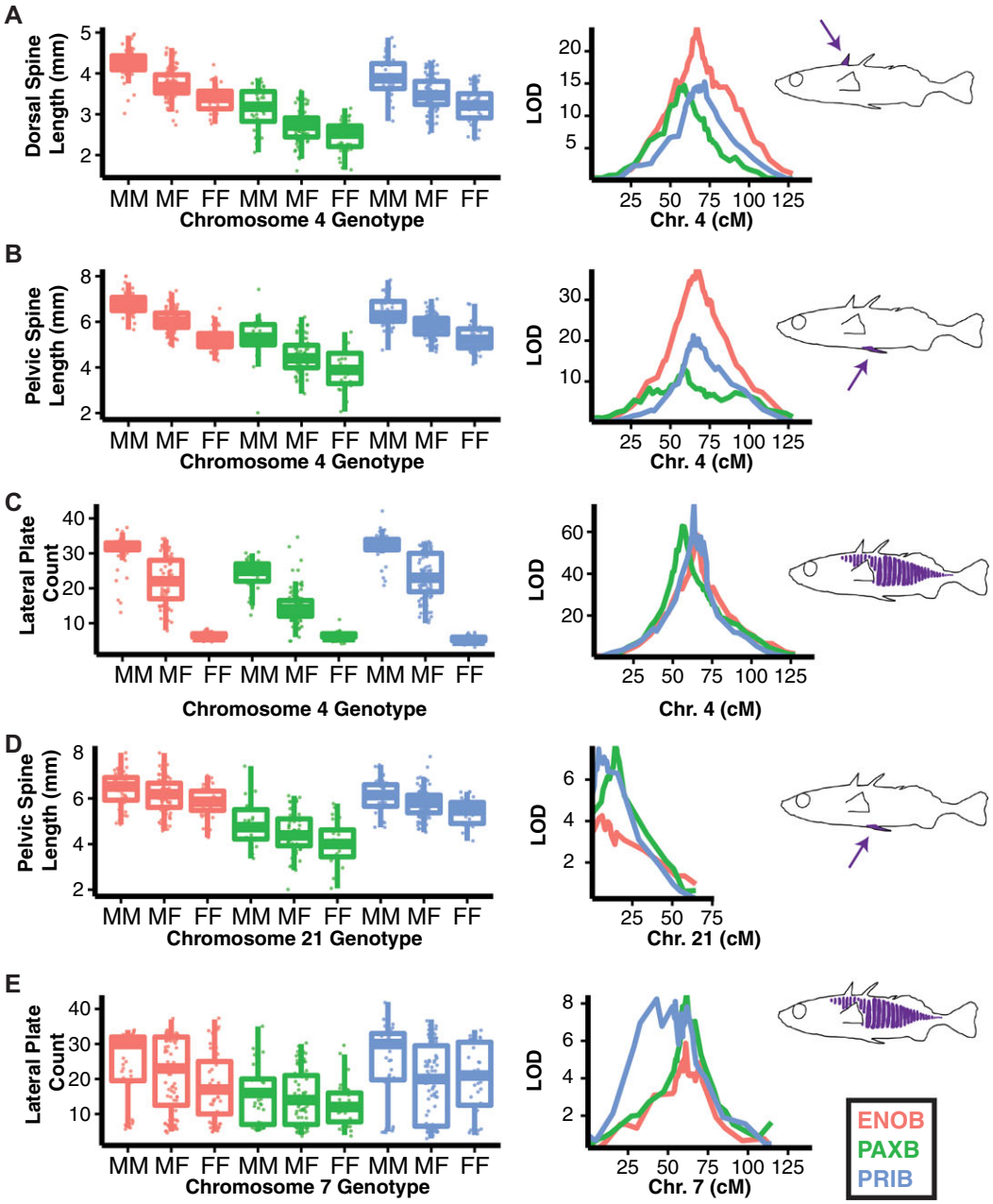

Figure 3. Armor QTL identified in all three crosses at the genome-wide (LOD 3.7) significance level. Phenotypes for each trait are shown for fish with MM, MF, or FF genotypes at the peak marker in each cross on the left, where M = marine allele and F = freshwater allele. For all five QTL, the freshwater allele produces smaller or fewer skeletal elements. The LOD profiles of each QTL plotted relative to genetic distance (cM) are shown in the middle. Since genetic distance varies between crosses, the position of each marker was scaled relative to the total mean genetic length of the chromosome in all three crosses. Cartoon illustrations of armor phenotypes measured or counted are on the right, with skeletal elements highlighted in purple. (A) Dorsal spine 1 length, chr. 4; (B) Left pelvic spine length, chr. 4; (C) Lateral plate count, chr. 4; (D) Left pelvic spine length, chr. 21; (E) Lateral plate count, chr. 7. PAXB = green, PRIB = blue, ENOB = red. See Table S4 for additional information about each QTL.

significant parallelism when we account for potential pleiotropy on chromosomes 4 and 21. As a second, even more conservative, control for the potential pleiotropy of QTL, we performed an additional filtering in our simulations that allows a genomic position to be covered by at most one skeletal QTL per cross, regardless of trait (see supplemental methods). Although five genomic regions contained a QTL affecting at least one trait in all three crosses, neither double nor triple QTL were statistically significantly enriched relative to the expectations from these simulations (see Table S6).

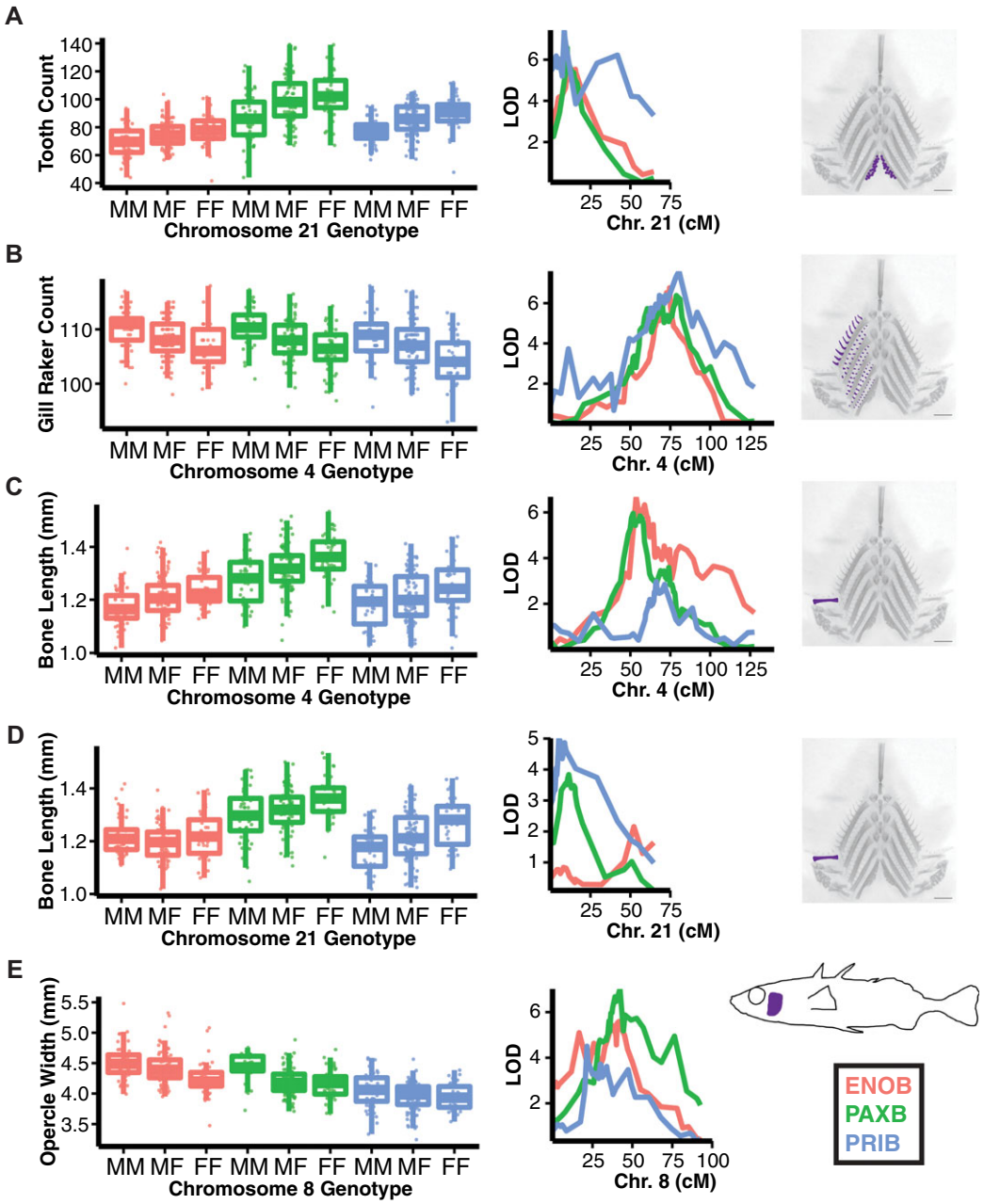

Figure 4. Shared craniofacial QTL. QTL for gill raker number (chr. 4), ventral pharyngeal tooth number (chr. 21), and branchial bone length (chr. 4 and chr. 21) have previously been described in PAXB × marine crosses (Cleves et al. 2014; Erickson et al. 2014; Glazer et al. 2014; Miller and Glazer et al. 2014). Phenotypes for each genotypic class at the peak marker are shown on the left (M = marine, F = freshwater). The LOD profiles of each QTL plotted relative to genetic distance (cM) are shown in the middle, as in Figure 3. Craniofacial bones measured or counted are highlighted in purple to the right; images of flat-mounted branchial skeletons are shown with anterior at top. Panels A, B and E show triple-overlapping QTL at the genome-wide threshold; C and D each include one suggestive parallel QTL. (A) Ventral pharyngeal teeth, chr. 21; (B) All row 1–9 gill rakers, chr. 4; (C) Epibranchial 1 length, chr. 4; (D) Epibranchial 1 length, chr. 21; (E) Opercle width, chr. 8. PAXB = green, PRIB = blue, ENOB = red.

We might fail to detect some overlapping QTL due to small effect sizes that fail to meet the strict genome-wide LOD cutoff (Beavis 1998). To test this possibility, we performed a second search for QTL by looking for suggestive parallel QTL in the regions of the genome where QTL had been identified in at least one cross for the trait class. This analysis identified a total of 43 new suggestive QTL (13 PAXB, 13 PRIB, and 17 ENOB, Fig. S4, Fig. S5, Table S5), including six new triple-overlapping QTL that were not previously identified at the genome-wide significance threshold (Fig. 2B, Table S9). We found that 68% and 71% of

Table 1. Results of QTL overlap simulation.

QTL type	Observed	Mean simulated	2.5% simulated	97.5% simulated	*P* (simulated ≥ observed)
PAXB unique	18	24.2	19	29	0.9964
PRIB unique	24	29.9	25	35	0.994
ENOB unique	15	19.4	15	23	0.9826
PAXB-PRIB overlapping	6	5.6	2	10	0.5014
PAXB-ENOB overlapping	2	2.9	0	6	0.8149
PRIB-ENOB overlapping	2	4.3	1	8	0.9477
triple overlapping	8	0.6	0	2	**<0.0001**

The physical locations of the QTL were randomly permuted 10,000 times and tested for overlap between crosses in each permutation. The total number of pairwise overlaps and triple overlaps was counted. The mean, 2.5 and 97.5 percentiles of permuted overlapping QTL are presented. The *P*-value was calculated based on the number of permutations with an equal or greater number of overlaps than the actual observed overlaps. See Fig. S3 for the distribution of simulated numbers of QTL overlaps.

the suggestive double-and triple-overlapping QTL, respectively, had effects in the same direction. When these QTL are included, 67% of all QTL overlap with at least one other QTL, and 33% are unique to a single cross. Thus, the relatively small sizes of our crosses (∼180 F2s) may have prevented us from detecting some overlapping QTL, causing us to underestimate parallelism in the main analysis.

MOST QTL ARE NOT SHARED BETWEEN LAKES

Despite the significant enrichment for overlap of genomic regions influencing similar phenotypes in the three crosses, the majority of QTL identified (57%) were not shared. Several striking nonparallel genetic patterns were observed. For example, severe pelvic reduction has been described in Paxton benthics, but not in Priest or Enos benthics. Consistent with the previously described role of *Pitx1* in mediating pelvic reduction in PAXB fish (Shapiro et al. 2004; Chan et al. 2010), we detected a large effect QTL controlling presence or absence of pelvic spines in the PAXB cross that mapped to the end of chromosome 7 containing *Pitx1* (Fig. S6). Over half of all gill raker QTL (9/16) were unique to a single cross, and of 13 QTL affecting branchial bone length, only one double-overlapping QTL was observed at the genome-wide significance cutoff.

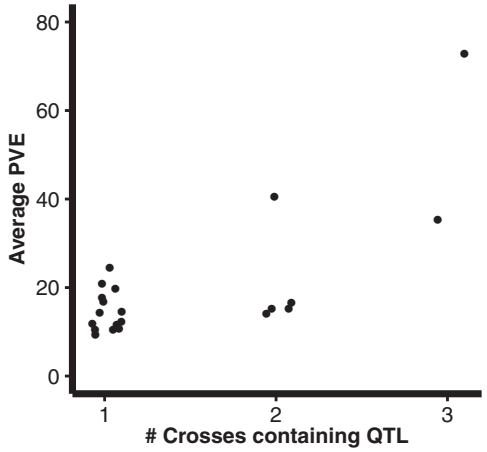

Figure 5. Larger effect QTL overlap in multiple benthic crosses. The highest PVE QTL controlling each trait class, including suggestive QTL, was identified for each cross to reduce oversampling of phenotypes with multiple QTL. QTL were classified as overlapping in 1, 2, or 3 crosses based on overlap of the physical 1.5 LOD intervals for QTL controlling the same trait category. For overlapping QTL, the average PVE was calculated between crosses. Average PVE is plotted against the number of crosses in which overlapping QTL were found, and points are jittered along the *x*-axis to show all points. A significant relationship between effect size and parallelism was observed (Spearman rank correlation, $\rho = 0.50$, $n = 20$, $P = 0.02$).

WEAK RELATIONSHIP BETWEEN QTL EFFECT SIZE AND PARALLELISM

We tested the prediction of Conte et al. (2015) that large effect QTL would be more likely to overlap in multiple benthic populations than small effect QTL. Briefly, population genetics theory predicts that evolution via new mutations or standing variation should produce a positive correlation between parallelism and QTL effect size. We tested this prediction by examining the relationship between the average PVE of each QTL within a trait class and the number of overlaps for that QTL. Because traits with many small effect QTL might have QTL that overlap by chance, we restricted our analysis of PVE versus effect size to only the largest effect QTL affecting each trait class in each cross, to reduce oversampling of trait categories with many QTL (as in Conte et al. 2015). Because small effect QTL are less likely to be detected in parallel, we included suggestive QTL if they were the largest effect QTL for the trait. We found a significant relationship between PVE and effect size ($\rho = 0.50$, $n = 20$, $P = 0.02$, Fig. 5), though this effect was driven by the

large effect, parallel QTL on chromosome 4. When we performed this correlation analysis on the list of maximally filtered QTL regions, to reduce oversampling of individual chromosomes, the relationship was not significant ($\rho = 0.139$, $n = 28$, $P = 0.49$). Therefore, our results confirm those of Conte et al. (2015)—the relationship between parallelism and effect size is at most weak and driven by a few QTL on the same chromosome.

BENTHIC QTL ARE CLUSTERED IN THE GENOME

In a previous PAXB × marine cross, three chromosomes (4, 20, and 21) were enriched for QTL for the studied traits (Miller et al. 2014). We hypothesized that enrichment of QTL on these chromosomes is a general feature of adaptation to freshwater benthic environments. We tested for significant clustering of QTL in all three crosses using a chi-square test and null expectations based on physical length, genetic length, and Ensembl-predicted gene number (using the revised assembly from Glazer et al. 2015). We found that, by all three expectations, the PAXB cross was enriched for QTL on chromosome 21, the PRIB cross was enriched for QTL on chromosomes 4 and 21, and the ENOB cross was enriched for QTL on chromosome 4 (Table 2). Additionally, enrichment for QTL on chromosomes 19 and 5 was seen in the PRIB and ENOB crosses, respectively, for two of these three tests of clustering. Therefore, two of the three previously identified trait clusters were found in benthic populations other than PAXB and had significantly clustered QTL on chromosomes when accounting for chromosome genetic length, physical length, or gene number. Interestingly, using the same chi-square test to compare predicted gene number on each chromosome to its physical length based on the revised genome assembly of Glazer et al. 2015, we found that chromosomes 4 and 21 have significantly fewer genes than expected based on their physical length (standard residuals of –5.70 and –7.61, respectively). Despite this low gene number, they have more QTL per unit physical length.

BENTHIC QTL ARE ENRICHED FOR GENOMIC SIGNATURES OF NATURAL SELECTION

Next, we hypothesized that QTL important for benthic adaptation would be enriched for loci showing signatures of natural selection in two analyses of stickleback divergence: marine-freshwater (Jones et al. 2012b) and benthic-limnetic (Jones et al. 2012a). These studies looked for genetic variants that were shared among freshwater (or benthic) populations and differed from marine (or limnetic) populations. We tested for enrichment by calculating the number of selected loci overlapping with benthic skeletal QTL compared to a randomly permuted set of QTL. We found that shared QTL (double- and triple-overlapping) were enriched for loci showing marine-freshwater signals of selection ($P < 0.05$, Table 3). However, unshared QTL, those found in single lakes, were not enriched ($P = 0.18$–0.54, Table 3). Shared QTL were also enriched for a set of 46 SNPs found to have high F_{ST} in at least one benthic-limnetic species pair (Jones et al. 2012a), whereas the unshared, lake-specific QTL were not enriched (with the exception of Paxton lake, which was slightly enriched, Table 3). When we included the suggestive parallel QTL in the analysis, the results were similar, with double- and triple-overlapping QTL enriched for both marine-freshwater and benthic-limnetic signals of selection (Table S7). Thus, the overlapping genomic regions underlying similar skeletal QTL are enriched for loci showing population genetic signals of selection, suggesting these genomic regions are under strong natural selection during benthic adaptation.

MOST QTL DO NOT OVERLAP THREE PREVIOUSLY IDENTIFIED INVERSIONS

Chromosomal inversions are theoretically predicted to contribute to adaptation when there is gene flow and multiple loci with selected alleles are found within the inversion (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008). Supporting these predictions, inversions contribute to evolved differences between populations of butterflies, sparrows, and monkeyflowers (Thomas et al. 2008; Lowry and Willis 2010; Joron et al. 2011; Fishman et al. 2013; Kunte et al. 2014). In sticklebacks, three chromosomal inversions typically have different orientations in marine and freshwater populations (Jones et al. 2012b). A total of 11 detected QTL overlap one of these three inversions, a significant enrichment relative to QTL placed randomly in the genome ($P = 0.02$, based on 10,000 permutations). This enrichment was driven mainly by the QTL cluster on chromosome 21 (Fig. S5). We have evidence that at least some of the QTL overlapping this inversion genetically map outside of the inversion (see Discussion).

Discussion

PARALLEL QTL ARE ENRICHED, BUT THE MAJORITY OF QTL ARE NONPARALLEL

The benthic-limnetic stickleback species pairs provide a powerful system to study ecological adaptation and incipient speciation (Schluter and Rambaut 1996; Schluter 2001). One long-standing question has been the extent of genetic parallelism underlying the benthic and limnetic phenotypic convergence across lakes (Schluter and Conte 2009; Schluter et al. 2010). Prior to this study, the only study addressing the extent of genetic parallelism underlying phenotypic convergence in this system used QTL mapping in benthic-limnetic crosses from two lakes containing species pairs (Conte et al. 2015). Here, we significantly extend our understanding of the genetic basis of convergent adaptation by studying the benthic ecotype from three lakes with species pairs and using a common marine genetic background. To our knowledge, this study represents one of the first to use genome-wide linkage

Table 2. Results of QTL clustering analyses.

	By genes (Ensembl)		By physical length (Mb)		By genetic length (cM)	
Cross	*P*	Enriched Chr.	*P*	Enriched Chr.	*P*	Enriched Chr.
PAXB	0.004	21	0.04	21	0.01	21
PRIB	0.008	4, 19, 21	0.034	4, 19, 21	0.019	4, 21
ENOB	0.008	4	0.018	4, 5	0.005	4, 5

A chi-square test was used to test whether the distribution of QTL across chromosomes was proportional to gene number (based on Ensembl predictions), physical length (Mb), or genetic length (cM). *P*-values were based on 10,000 permutations of the data, and enriched chromosomes are chromosomes that had standard residuals > 2 (as in Arnegard et al. 2014).

Table 3. Shared QTL are enriched for marine-freshwater and benthic-limnetic genomic signals of selection.

	Signals of selection:	
	Marine-freshwater	Benthic-limnetic
QTL set:		
PAXB unique (n = 18)	1.36 (0.18)	1.85 (**0.03**)
PRIB unique (n = 24)	1.15 (0.28)	1.34 (0.11)
ENOB unique (n = 15)	0.88 (0.54)	0.99 (0.44)
All double (n = 10)	2.24 (**0.0011**)	1.91 (**0.002**)
All triple (n = 8)	3.59 (**<0.0001**)	2.35 (**0.0006**)

The number of overlaps between loci with signals of selection and benthic QTL were counted and compared to 10,000 random permutations of the QTL locations. Values given are the fold enrichment followed by the *P*-value in parentheses. Marine-freshwater loci with signals of selection based on Jones et al. 2012b and benthic-limnetic loci with signals of selection based on Jones et al. 2012a.

mapping in three independently derived, convergently evolved lineages to study the genetic basis of repeated adaptive divergence.

We found that the genomic regions underlying benthic adaptation in three independently derived populations significantly overlap, supporting the hypothesis of a parallel genetic component to convergent skeletal evolution. We found that 47% (16/33) of PAXB QTL, 40% (16/40) of PRIB QTL, and 44% (12/27) of ENOB QTL overlapped a QTL affecting a similar phenotype in at least one other benthic population. Furthermore, eight QTL underlying similar phenotypes, accounting for 20–29% of all QTL found in each cross, overlapped in all three populations, with 88% having effects in the same direction in all three crosses. It is important to note that since all QTL identified here contain multiple genes, finding overlapping QTL does not necessarily mean that the underlying genes or genetic changes are the same. The genetic resolution of these QTL is coarse (due to a small cross size of ~180 F2s per cross), and many QTL regions contain hundreds of genes. These results suggest that some shared large genomic regions repeatedly underlie benthic adaptation. However, only identifying the actual genes underlying these evolved phenotypes can answer the question of whether true genetic parallelism has occurred.

Despite this uncertainty of whether QTL parallelism reflects genetic parallelism, observing nonoverlapping QTL is more straightforward to interpret, as nonoverlapping QTL strongly support a nonparallel genetic basis. In this study, despite the significant enrichment for overlapping QTL, 57% of all detected QTL were found in only one population. This partially predictable but largely nonparallel basis of convergent evolution is consistent with previous findings that although 35% of divergent genomic regions within a single marine-freshwater contrast were shared with marine-freshwater divergence worldwide, 65% were not (Jones et al. 2012a). Furthermore, our results of partially repeatable evolution are consistent with the degree of genetic parallelism found in a previous study of benthic and limnetic sticklebacks (Conte et al. 2015) as well as a meta-analysis of parallelism in a wide variety of organisms (Conte et al. 2012). Our results highlight a remarkable outcome: benthic adaptation has occurred three times via largely different genetic mechanisms. The largely nonparallel genetic basis for dozens of phenotypes suggests that previous findings on sticklebacks that identified a parallel genetic basis for freshwater traits (Colosimo et al. 2004, 2005; Cresko et al. 2004; Miller et al. 2007; Chan et al. 2010) are not representative of all traits.

Our results showing a partially predictable genomic basis of convergent evolution fit within a spectrum of previous work showing both repeated and nonrepeated bases of convergent evolution at the QTL level in diverse organisms. Three species of *Mimulus* have all convergently evolved changes in leaf shape. In all three species, leaf shape mapped to the same two genomic regions (Ferris et al. 2015). Likewise, multiple populations of clinally adapted *Drosophila melanogaster* share overlapping QTL for wing size (Gockel et al. 2002; Calboli et al. 2003). However, in *D. simulans*, similar clinal variation in wing size maps to one genomic region that overlaps a *melanogaster* QTL and one distinct region (Lee et al. 2011). Similar to our finding, two strains of weedy rice have adapted to the agricultural environment through

nonoverlapping QTL for three traits, but partially overlapping QTL for a fourth trait (Thurber et al. 2013). Thus our work adds to a growing list of partial parallelism at the QTL level, suggesting that the same genomic regions only sometimes underlie convergent adaptation, and enabling future work to test genetic parallelism.

PLEIOTROPY, QTL CLUSTERING, AND INVERSIONS

Pleiotropic loci influencing multiple adaptive phenotypes have been observed in monkeyflowers, rice, flies, and mice (Hall et al. 2006; Yan et al. 2011; Linnen et al. 2013; Paaby et al. 2014) and could explain the multiple triple-overlapping QTL found on chromosome 4. The peak marker and 1.5 LOD intervals were highly similar for the chromosome 4 pelvic and dorsal spine length QTL within each cross, and both overlapped a gill raker QTL and the previously described *Eda* lateral plate QTL (Colosimo et al. 2004, 2005), which was found in all three crosses. Parsimoniously, a shared locus with pleiotropic effects could underlie all four phenotypes in benthic populations. Although the *Eda* haplotype that controls lateral plates has not been reported to affect gill raker or spine morphology, *Eda* mRNA expression is observed in dorsal and pelvic spine tissue (Colosimo et al. 2004, 2005; O'Brown et al. 2015), *Eda receptor* expression is detected in forming gill raker buds (Glazer et al. 2014), and zebrafish *Eda* mutants lack dorsal fins, pelvic fins, and gill rakers (Harris et al. 2008). However, in PRIB, the dorsal spine QTL does not overlap *Eda*, and in PAXB, the gill raker QTL does not overlap any other QTL, suggesting these traits are affected by separate tightly linked loci. Thus, we hypothesize chromosome 4 has at least two armor reduction loci (*Eda* plus at least one spine length locus), as well as a gill raker reduction locus, that form a skeletal reduction supergene (Schwander et al. 2014). Identifying the genes underlying these QTL and testing whether freshwater alleles are in linkage disequilibrium with the low-armor *Eda* allele in marine fish could test this hypothesis. These results combined with the overall genomic clustering of QTL suggest an important role for pleiotropic QTL and/or supergenes as drivers of parallel adaptation in benthic environments. Approaches to infer pleiotropy in QTL studies have been developed (Jiang and Zeng 1995), which future analyses could apply to correlated traits in sticklebacks.

Like previous studies, (Arnegard et al. 2014; Liu et al. 2014; Miller et al. 2014) we mapped multiple QTL to chromosomes 4 and 21 in some crosses. Unlike Miller et al. (2014), we did not identify a chromosome 20 QTL cluster, perhaps because the PAXB grandparent used in this cross and the grandparent used in the previous cross had different chromosome 20 alleles. Additionally, the relatively smaller sizes of our cross and reduced number of phenotypes scored may have prevented us from detecting some loci that contribute to clustering (e.g., we did not phenotype several bones that were part of the cluster found in Miller et al. 2014). Furthermore, because clustering was seen when we adjusted for genetic length, physical length, or gene number, clustering does not appear to result simply from recombination suppression or differential gene density between chromosomes.

Theoretical work has proposed that inversions could evolve because they cluster adaptive loci and prevent their recombination (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008). In sticklebacks, inversions on chromosomes 1, 11, and 21 are oppositely fixed in most marine and freshwater populations including the PAXB population (Jones et al. 2012b). These three inversions were significantly enriched within detected QTL intervals, including two triple QTL and two double QTL on chromosome 21. However, in the PAXB population, tooth number fine-maps to a genomic interval over a megabase outside of the inversion (Cleves et al. 2014), so the triple-overlapping tooth number QTL is likely not in the inversion. Likewise, the PAXB pelvic spine length QTL also maps entirely outside the inversion, so the triple-overlapping pelvic spine length QTL is also unlikely to be in the inversion. Chromosome 4, highly enriched for QTL and triple-overlapping QTL, does not contain one of these inversions. Therefore, although a few QTL could be due to mutations within inversions, most QTL involved in benthic skeletal adaptation (at least 89/100, since only 11/100 QTL overlap one of these three inversions) do not map to these three previously described inversions.

SHARED QTL AND FRESHWATER ADAPTATION

Many of the triple QTL we identified have been found in previous studies, but little was known about their parallelism. A chromosome 4 gill raker reduction QTL was found in three freshwater populations (Glazer et al. 2014) and was detected in all three benthic populations here. A chromosome 21 tooth QTL maps to a *cis*-regulatory allele of the *Bmp6* gene in the PAXB population (Cleves et al. 2014), and the presence of a similar tooth gain QTL in two other benthic populations suggests that increased tooth number is adaptive in benthic environments. Chromosomes 4 and 21 also increase branchial bone length in multiple freshwater populations (Erickson et al. 2014), and each QTL was found in a second benthic population, suggesting increased branchial bone length may also be adaptive. Continued mapping of these QTL will help determine whether the parallelism we observe is due to different tightly linked genes, different mutations in the same gene, or repeated selection of standing variants. Given the geographic proximity of the lakes, we hypothesize that common shared ancestral alleles underlie the shared QTL, whereas QTL unique to a single cross are due to rarer ancestral variants or new mutations.

Genomic techniques such as RAD-seq and genome sequencing have enabled the discovery of local and temporal genomic signatures of selection in a variety of organisms including maize (Hufford et al. 2012), monkeyflowers (Stankowski and Streisfeld

2015), flies (Bergland et al. 2014), stick insects (Soria-Carrasco et al. 2014), wolves (Schweizer et al. 2015), cichlids (Ford et al. 2015), whitefish (Laporte et al. 2015), salmon (Seeb et al. 2014), and sheep (Kardos et al. 2015). However, an understanding of the phenotypes underlying these regions is often far more limited. QTL mapping of diverse phenotypes that differ between populations can provide a starting point to connect genomic signatures of selection to loci affecting morphology and physiology. We found that overlapping QTL were strongly enriched for genomic signatures of recurrent natural selection in multiple freshwater populations (Jones et al. 2012b). Importantly, these genomic regions are divergent in multiple freshwater and marine populations, so the QTL enrichment in these regions may be related to skeletal changes involved in general freshwater adaptation, rather than benthic adaptation. However, shared QTL were also enriched for SNPs that are F_{ST} outliers between limnetic and benthic fish (Jones et al. 2012a), suggesting some of the QTL might underlie benthic adaptation. Whether the QTL we identified are due to genetic variants that drive these signals of selection or whether the QTL are hitchhiking along with other loci important for freshwater adaptation remains currently unknown. However, the signals of selection can pinpoint interesting candidate genes for the QTL intervals, which ultimately could link these population genetic signals of selection to adaptive phenotypes.

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DATA ARCHIVING

All sequence reads are deposited in the Sequence Read Archive (accession number SRP070856).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Methods
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Table S9. (.xlsx file) Locations of all double and triple QTL detected including suggestive QTL.
Supplemental File 1. (.xlsx file) All data used for QTL mapping.