Adaptive Evolution of Pelvic Reduction in Sticklebacks by Recurrent Deletion of a Pitx1 Enhancer

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The molecular mechanisms underlying major phenotypic changes that have evolved repeatedly in nature are generally unknown. Pelvic loss in different natural populations of threespine stickleback fish has occurred through regulatory mutations deleting a tissue-specific enhancer of the Pituitary homeobox transcription factor 1 (Pitx1) gene. The high prevalence of deletion mutations at Pitx1 may be influenced by inherent structural features of the locus. Although Pitx1 null mutations are lethal in laboratory animals, Pitx1 regulatory mutations show molecular signatures of positive selection in pelvic-reduced populations. These studies illustrate how major expression and morphological changes can arise from single mutational leaps in natural populations, producing new adaptive alleles via recurrent regulatory alterations in a key developmental control gene.

Evolutionary biology has been animated by long-standing debates about the number and type of genetic alterations that underlie evolutionary change. Questions about the roles of genetic changes of infinitesimally small versus large effects, the origin of traits by either natural selection or genetic drift, and the relative importance of coding and regulatory changes in evolution are currently being actively investigated (1–4). One of the classic examples of major evolutionary change in vertebrates is the extensive modification of paired appendages seen in different species (5). Although essential for many forms of locomotion, paired appendages have also been repeatedly lost in some fish, amphibian, reptile, and mammalian lineages, probably via selection for streamlined body forms (6).

Threespine stickleback fish (Gasterosteus aculeatus) make it possible to analyze the evolution, genetics, and development of major skeletal changes in natural populations (7). The pelvic apparatus of marine sticklebacks consists of prominent serrated spines that articulate with an underlying pelvic girdle that extends along the ventral and lateral sides of the fish (inspiring the scientific name Gasterosteus aculeatus, or bony stomach with spines). Although most sticklebacks develop a robust pelvic apparatus, over two dozen times widely distributed and probably independent freshwater stickleback populations show partial or complete loss of pelvic structures (8). Several factors may contribute to repeated evolution of pelvic reduction, including the absence of gape-limited predatory fish, limited calcium availability, and predation by grasping insects (9–12).

Genome-wide linkage mapping has identified a single chromosomal region that explains more than two thirds of the variance in pelvic size in crosses with pelvic-reduced sticklebacks (13–15). This region contains Pituitary homeobox 1 (Pitx1), a gene expressed in hindlimbs but not forelimbs of many different vertebrates and required for normal hindlimb development (13). Although the Pitx1 gene of pelvic-reduced sticklebacks shows no protein-coding changes as compared with that of ancestral marine fish, its expression in the developing pelvic region is almost completely lost (13, 16). On the basis of the map location, changes in expression, and directional asymmetry shared in both Pitx1-null mice and pelvic-reduced sticklebacks, cis-regulatory mutations at the Pitx1 locus have been proposed as the basis of stickleback pelvic reduction (13). However, regulatory mutations are difficult to identify, and the actual sequences controlling pelvic reduction have remained hypothetical (2).

Cis-regulatory changes at Pitx1 locus. Although Pitx1 represents a strong candidate gene for pelvic reduction, other genes in the larger chromosome region could be the real cause of pelvic loss, leading to secondary or trans-acting reduction of Pitx1 expression (2). To test this possibility, we generated F1 hybrids between pelvic-complete [Friant Low (FRIL) and pelvic-reduced (Paxton Lake Benthic (PAXB))] sticklebacks (see table S1 for geographic location of all populations used in this study (17)). F1 hybrid fish develop pelvic structures and contain both Pitx1 alleles in an identical trans-acting environment. The PAXB allele was expressed at significantly lower levels than the FRIL allele in the restored pelvic tissue of F1 hybrids (n = 19 individuals, two-tailed t test, P < 0.001) (Fig. 1). Reduced expression of the PAXB allele was tissue-specific because both Pitx1 alleles were expressed at similar levels in F1 hybrid head tissue. As a control, we generated F1 hybrids between two pelvic-complete populations (FRIL and Little Campbell River (LITC)) (Fig. 1). In this cross, both Pitx1 alleles were expressed at comparable levels in both heads and
pelvis. Allele-specific down-regulation of Pitx1 in the FRIL × PAXB cross shows that pelvic-specific loss of Pitx1 expression is due to cis-regulatory change (or changes) at Pitx1 itself and not to overall failure of pelvic development or changes in unknown trans-acting factors.

**Fine mapping of pelvic regulatory region.** To further localize the position of the cis-acting changes, we looked for the smallest chromosomal region co-segregating with bilateral absence of pelvic structures in a cross between pelvic-complete [Japanese marine (JAMA) and pelvic-reduced (PAXB) fish (13)]. High-resolution mapping identified a 124-kb minimal interval, containing only the Pitx1 and Histone 2A (H2AFY) genes, which showed perfect concordance between PAXB alleles and absence of the pelvis (fig. S1A).

Recombination in natural populations can also be used to narrow the size of regions controlling polymorphic traits in sticklebacks (18). We therefore tested whether markers in the Pitx1 region were associated with the presence or absence of pelvic structures in lakes with dimorphic stickleback forms: benthic and limnetic sticklebacks from Paxton Lake, British Columbia (PAXB/PAXL), and pelvic-complete and pelvic-reduced sticklebacks from Wallace Lake, Alaska (WALR/WALC) (fig. S2) (13, 14). Microsatellite markers located in an intergenic region approximately 30 kb upstream of Pitx1 showed highly significant allelic frequency differences in fish with contrasting pelvic phenotypes ($P < 10^{-33}$) (Fig. S1B and table S2). In contrast, markers around the Pitx1 and H2AFY coding regions showed little or no differentiation above background levels. These results suggest that an approximately 23-kb intergenic region upstream of Pitx1 controls pelvic development. This region is conserved among zebrafish and other teleosts (Fig. 2A), suggesting that it may contain ancestrally conserved regulatory enhancers.

**A small enhancer drives pelvic expression of Pitx1.** To test for regulatory functions in the Pitx1 intergenic region, we cloned different subfragments upstream of a basal promoter and enhanced green fluorescent protein (EGFP) reporter gene (Fig. 2B) (19). The hsp70 promoter drives modest or no EGFP expression except in the eye (19). A construct containing a 2.5-kb fragment from a marine, pelvic-complete fish (Salmon River (SALR)) drove consistent EGFP expression in the developing pelvic region of transgenic sticklebacks (four of five independent transgenics) (Fig. 2, C and F). A smaller 501-bp (bp) subfragment also drove highly specific pelvic expression (seven of nine transgenics) (Fig. 2, E and H). No consistent expression was seen in pectoral fins or other sites of normal Pitx1 expression, including the mouth, jaw, and pituitary (13, 16). Thus, the noncoding region upstream of Pitx1 contains a tissue-specific enhancer for hindfin expression, which we term “Pel.” Pel shows sequence conservation across distantly related teleost fish (Fig. 2A and fig. S3) and contains multiple predicted transcription factor binding sites that might contribute to spatially restricted expression in the developing pelvic region (fig. S4).

**Transgenic rescue of pelvic reduction.** If regulatory changes in Pitx1 underlie pelvic reduction in sticklebacks, restoring pelvic expression of Pitx1 should rescue pelvic structures. We cloned the 2.5-kb Pel region from a pelvic-complete population (SALR) upstream of a Pitx1 minigene that was prepared from coding exons of a pelvic-reduced fish [Bear Paw Lake (BEPA)] (14). The transgenic rescue construct was injected into fertilized eggs of BEPA fish, which normally fail to develop any pelvic spine and show no more than a small vestigial remnant of the underlying pelvic girdle: pelvic score $\leq 3$ (Fig. 3, F, G, and S5D) (12). Transgenic fry showed variable but enhanced development of external pelvic spines as com-

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**Fig. 2.** (A) VISTA/mLAGAN (http://genome.lbl.gov/vista/) alignment of Pitx1 candidate region from pelvic-complete stickleback (SALR), medaka, and zebrafish. Red peaks indicate $>40\%$ sequence identity in 20-bp sliding windows; grey bars at top indicate repetitive sequences; and circles indicate microsatellite markers used in association mapping in fig. S1. (B) Reporter gene expression in transgenic animals. (C) Pel-2.5-kbSALR from a marine population drives tissue-specific EGFP (green) expression in the developing pelvic bud of Swarup stage-32 larvae (36). (F) Detail of (C). (D and G) Altered Pel-$\Delta$2.5-kbPAXB sequence from pelvic-reduced PAXB stickleback fails to drive pelvic EGFP expression. (E and H) A smaller fragment from marine fish, Pel-501-bpSALR, also drives EGFP expression in the developing pelvic bud of multiple stage-30 larvae. This region is completely missing in PAXB.

**Fig. 3.** (A) Juvenile pelvic-reduced BEPA stickleback expressing a Pitx1 transgene driven by the Pel-2.5-kbSALR enhancer compared with (B) uninjected sibling. External spines form only in transgenic fish (arrowhead). (C and D) Alizarin red–stained pelvic structures of adult transgenic fish compared with BEPA parental phenotype. BEPA fish normally develop only a small ovoid vestige (OV) of the anterior pelvic process (AP). Transgenic fish show clear development of the AP, ascending branch (AB), and posterior process (PP) of the pelvis, and a prominent serrated pelvic spine. Pectoral fin (PF) rays develop in both fish.
pared with those of control un.injected siblings (clutch 1, n = 16 injected and 11 uninjected fish, Wilcoxon rank-sum test, W = 1073.5, P < 0.01; clutch 2, n = 4 injected and 18 uninjected fish, W = 513, P = 2.3 × 10^{-5}) (Fig. 3A). Alizarin red skeletal preparations of two adult transgenic fish revealed prominent serrated spines articulating with an enlarged, complex pelvic girdle containing anterior, posterior, and ascending branch structures (Fig. 3C and fig. S5, pelvic score summary). These data provide functional evidence that Pel-Pitx1 is a major determinant of pelvic formation in sticklebacks.

**Nature of mutations in pelvic-reduced fish.** Bacterial artificial chromosome sequencing from the PAXB population identified a 1868-bp deletion present in the PAXB population identified a 1868-bp deletion, confirming that the molecular deletion present in the pelvic-reduced population identified a 1868-bp deletion. For the PAXB, BEPA, and HUMP populations, the SNPs corresponding to the missing genotypes fall within the known deletion endpoints from DNA sequencing. The larger genotyping survey identified a total of nine different haplotypes with different staggered deletions, each consistently seen within a pelvic-reduced population, and each overlapping or completely removing the Pel enhancer region (Fig. 4 and fig. S8).

**Fragile sites.** Several features suggest that Pitx1 may be located within a fragile region of the genome: The gene is located at the telomeric end of linkage group 7; the region contains many repeats and failed to assemble in the stickleback genome; the enhancer region is difficult to amplify and sequence; and close inspection of the deletion boundaries in PAXB and BEPA revealed short 2- or 3-bp sequence identities present on both sides, one of which is retained after deletion (Fig. 4A and fig. S7A). Similar nested deletions and small sequence identities may occur by means of re-organization of chromosome ends after breakage and repair by nonhomologous end joining (NHEJ) (fig. S7B) (20, 21). In humans, NHEJ is associated with stalled replication forks at fragile chromosomal sites, which also are frequent in subtelomeric regions (21). Fragile sites are also enriched in sequences with high DNA flexibility, which is a physical property that can be calculated from known twist angles between different stacked DNA base pairs (20). DNA flexibility analysis of Pitx1 and the entire assembled stickleback genome showed a median flexibility score of 265 with a tail of extreme values. Four of the top 10 flexibility scores in the genome occur in the Pitx1 region, suggesting that this region is exceptionally flexible and may be prone to deletion (Wilcoxon rank sum = 59,624, P < 2 × 10^{-6}) (Fig. 4C).

**Signatures of selection.** Recurring deletions could explain how pelvic-reduction alleles arise repeatedly in widespread isolated populations. To test whether pelvic-reduction alleles have also been subject to positive selection, we looked for molecular signatures that commonly accompany selective sweeps, including reduced heterozygosity and an overrepresentation of derived alleles (22). Patterns of allelic variation showed an excess of derived alleles near the Pel enhancer region of pelvic-reduced populations, as indicated by negative values of Fay and Wu’s H statistic (Fig. 5A and fig. S9A) (23). We also observed a significant reduction in heterozygosity at or near the Pel enhancer in pelvic-reduced populations as compared with marine populations (two-tailed t test, P < 0.001) (Fig. 5, B and C). This reduction cannot be solely explained by population bottlenecks that occurred during freshwater colonization because heterozygosity reduction near Pel is specific to pelvic-reduced, but not pelvic-complete, freshwater populations (two-tailed t test, P < 0.002) (Fig. 5, B and C). In flanking regions of Pitx1, and in unlinked control loci, we observed no significant difference in heterozygosity between freshwater fish with a complete or missing pelvis (Fig. 5C). Pelvic-reduced populations were significantly more likely to exhibit minimum heterozygosity close to the Pel enhancer region than either marine or freshwater populations with a robust pelvis (two-tailed t test, P < 0.002) (fig. S9F). The local heterozygosity and H statistic minima around the Pel enhancer region suggest that changes in this region have been selected in pelvic-reduced stickleback populations.

**Fig. 4.** (A) SNP genotyping in additional pelvic-reduced populations identifies nine different deletions that overlap in a 488-bp region. Triangles indicate SNP markers; gray bars indicate putative deleted regions flanked by two failed SNP genotypes; dark blue bars indicate regions flanked by two successful SNP genotypes; light blue bars indicate regions with successful genotypes only on one side; red bars indicate positions of Pel-2.5-kb and Pel-501-bp enhancers. Apparent deletions were confirmed by means of sequencing in populations 4, 6, and 7, with the size of deletions indicated on the right, and micro-homologies of 2 to 3 bp at deletion junctions shown in red. (B) Location of populations surveyed. (C) TwistFlex (20) prediction of highly flexible DNA regions (red circles) in Pitx1 locus (Pel region score is 3263) compared with the frequency distribution of flexibility scores in the rest of the stickleback genome (median score is 265). The area of the red circles is proportional to the flexibility score.
Discussion. Traditional theories of evolution posit that adaptation occurs through many mutations of infinitesimally small effect. In contrast, recent work suggests that mutation effect sizes follow an exponential distribution, with mutations of large effect contributing to adaptive change in nature (1). We narrowed the candidate interval for a pelvic quantitative trait locus with large effects in sticklebacks to the noncoding region upstream of Pitx1 and identified a tissue-specific enhancer for pelvic expression that has been functionally inactivated in pelvic-reduced fish. Reintroduction of the enhancer and Pitx1 coding region can restore formation of pelvic structures in derived populations that appear to be monomorphic for pelvic reduction. The combined data from mapping, expression, molecular, transgenic, and population genetic studies illustrate how major morphological evolution can proceed through a regulatory change in a key developmental control gene.

Large evolutionary differences that map to a particular locus can still be caused by many linked small-effect mutations that have accumulated in that gene (24, 25). However, we find that pelvic-reduction in sticklebacks maps to a type of DNA lesion that may produce a large regulatory change in a single mutational leap: deletions that completely remove a regulatory enhancer. Smaller functional lesions might be found in some pelvic-reduced populations, including four populations without obvious deletions. However, three of these populations show unusual morphological features, suggesting that their pelvic loss may have occurred through non–Pitx1-mediated mechanisms (8, 26).

The Pitx1 locus scores as one of the most flexible regions in the stickleback genome, which may reflect a susceptibility to double-stranded DNA breaks and repair through NHEJ (27–29). We hypothesize that sequence features in the Pitx1 locus may predispose the locus to structural changes, possibly explaining the high prevalence of independent deletion mutations fixed in different pelvic-reduced stickleback populations. A similar spectrum of independent small-deletion mutations has been seen at the vernalization 1 locus of plants (30), suggesting that recurrent deletions in particular genes may also contribute to parallel evolution of other phenotypes in natural populations.

Mutations in developmental control genes are often deleterious in laboratory animals, leading to long-standing doubts about whether mutations in such genes could ever be advantageous in nature (31). Although Pitx1 coding regions are lethal in mice (32), we find clear signatures of positive selection in the Pitx1 gene of pelvic-reduced sticklebacks. Before this work, the primary evidence that pelvic reduction might be adaptive in sticklebacks came from repeated evolution of similar phenotypes in similar ecological environments and the temporal sequence of pelvic reduction in fossil sticklebacks (11, 12, 33). The molecular signatures of selection we have identified in the current study are centered on the tissue-specific Pel enhancer region rather than the Pitx1 coding region. Regulatory changes in developmental control genes have often been proposed as a possible basis for morphological evolution (3, 34). However, many proposed examples of regulatory evolution in wild animals have not yet been traced to particular sequences (2) or do not show obvious molecular signatures of selection in natural populations (35). Identification of the Pel enhancer underlying pelvic reduction in sticklebacks connects a major change in vertebrate skeletal structures to specific DNA sequence alterations and provides clear evidence for adaptive evolution surrounding the corresponding region in many different wild populations.

Fig. 5. (A and B) Fay and Wu’s H and relative heterozygosity (H) statistics across the Pitx1 region. Blue (freshwater pelvic-reduced) and green (freshwater pelvic-complete) data points and locally weighted scatterplot–smoothed (α = 0.2) line indicate the behavior in each group. The Pel-containing regulatory region of Pitx1 (gray candidate region [fig. S1B]) shows both negative H values, indicating an excess of derived alleles, and reduced heterozygosity in pelvic-reduced fish, which is consistent with positive selection. θ values are plotted relative to the grouped marine mean (per SNP) in order to control for variation in ascertainment between SNPs. (C) Heterozygosity (θ) from different genomic regions, grouped by population type. Freshwater fish show a general decrease in heterozygosity across both Pitx1 and control loci as compared with that of marine fish (red bars), as is expected from founding of new freshwater populations from marine ancestors. In the Pel enhancer region, but not in Pitx1-flanking regions or in control loci, pelvic-reduced freshwater populations (blue bars) show even lower heterozygosity than pelvic-complete freshwater populations (green bars) (**P < 0.01).

References and Notes
17. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material
www.sciencemag.org/cgi/content/full/science.1182213/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 to S5
References
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Adaptive Girdle Loss in Sticklebacks

How do molecular changes give rise to phenotypic adaptation exemplified by the repeated reduction in the pelvic girdle observed in separate populations of sticklebacks? Now Chan et al. (p. 302, published online 10 December) have identified the specific DNA changes that control this major skeletal adaptation. The key locus controlling pelvic phenotypes mapped to a noncoding regulatory region upstream of the Pituitary homeobox transcription factor 1 gene, which drives a tissue-specific pelvic enhancer. Multiple populations showed independent deletions in this region and enhancer function was inactivated. Reintroduction of the enhancer restored pelvic development in a pelvic-reduced stickleback.