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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*).

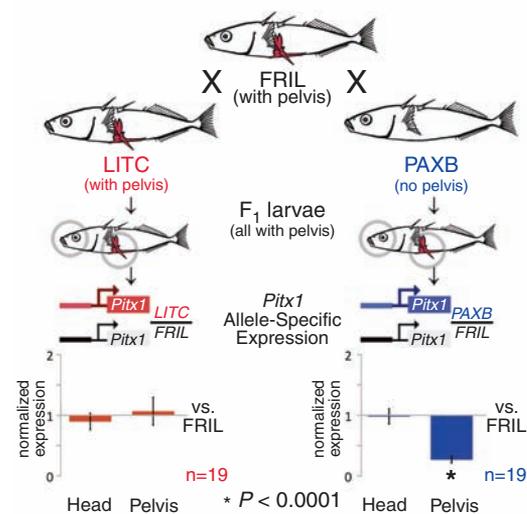Threespine stickleback fish (*Gasterosteus aculeatus*) make it possible to analyze the evolution, genetics, and development of major skel-etal changes in natural populations (*7*). The pelvic apparatus of marine sticklebacks consists of prominent serrated spines that articulate with an underlying pelvic 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 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 chromosome 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

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Fig. 1. Alleles of *Pitx1* from pelvic-complete (FRIL and LITC) and pelvic-reduced populations (PAXB) were combined in F1 hybrids, and brain and pelvic tissues were isolated so as to compare the expression of either the LITC or PAXB allele normalized to the level of expression of the FRIL allele in the same trans-acting environment. Expression of the PAXB *Pitx1* allele is greatly reduced in the pelvis but not the head of F1 hybrids (two-tailed t test, $P < 0.0001$), indicating a tissue-specific, cis-regulatory change in the *Pitx1* locus.

pelves. 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 chromo-some region co-segregating with bilateral ab-sence 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 be-tween PAXB alleles and absence of the pelvis (fig. S1A).
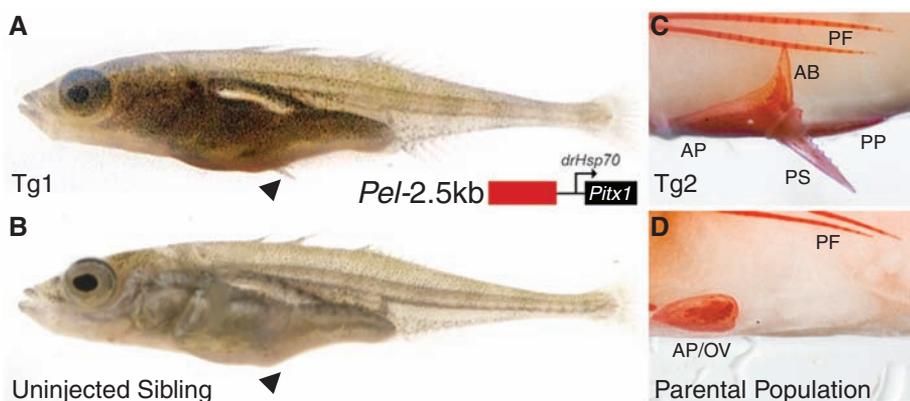
Recombination in natural populations can also be used to narrow the size of regions controlling polymorphic traits in sticklebacks (*18*). We there-fore tested whether markers in the *Pitx1* region were associated with the presence or absence of pelvic structures in lakes with dimorphic stickle-back forms: benthic and limnetic sticklebacks from Paxton Lake, British Columbia (PAXB/PAXL), and pelvic-complete and pelvic-reduced stickle-backs from Wallace Lake, Alaska (WALR/WALC) (fig. S2) (*13*, *14*). Microsatellite markers located in an intergenic region approximately 30 kb up-stream of *Pitx1* showed highly significant allele frequency differences in fish with contrasting pel-vic phenoytpes ($P < 10^{-35}$) (Fig. S1B and table S2). In contrast, markers around the *Pitx1* and *H2AFY* coding regions showed little or no differ-entiation above background levels. These results suggest that an approximately 23-kb intergenic region upstream of *Pitx1* controls pelvic devel-opment. This region is conserved among zebra-fish 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 sub-fragments upstream of a basal promoter and en-hanced 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 stick-lebacks (four of five independent transgenics) (Fig. 2, C and F). A smaller 501–base pair (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* ex-pression, 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 re-lated teleost fish (Fig. 2A and fig. S3) and contains multiple predicted transcription factor binding sites that might contribute to spatially restricted expres-sion in the developing pelvic region (fig. S4).

Transgenic rescue of pelvic reduction. If reg-ulatory changes in *Pitx1* underlie pelvic reduc-tion in sticklebacks, restoring pelvic expression of *Pitx1* should rescue pelvic structures. We cloned the 2.5-kb *Pel* region from a pelvic-complete pop-ulation (SALR) upstream of a *Pitx1* minigene that was prepared from coding exons of a pelvic-reduced fish [Bear Paw Lake (BEPA)] (*14*). The rescuing 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 ≤ 3) (Fig. 3, B and D, and fig. S5) (*12*). Transgenic fry showed variable but enhanced development of external pelvic spines as com-

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-kb^SALR 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*-Δ2.5-kb^PAXB sequence from pelvic-reduced PAXB stickleback fails to drive pelvic EGFP expression. (**E** and **H**) A smaller fragment from marine fish, *Pel*-501-bp^SALR, 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-kb^SALR 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 uninjected 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, $W = 513$, $P < 2.3 \times 10^{-9}$) (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 *Pel*-2.5-kb region (fig. S7). We cloned the PAXB-deleted variant and found that it no longer drove expression in the developing pelvis (zero out of eight transgenic animals) (Fig. 2, D and G), confirming that the molecular deletion in PAXB fish disrupts *Pel* enhancer function.

We also identified a second 757-bp deletion present in the pelvic-reduced BEPA population from Alaska and a third deletion of 973 bp present in the Hump Lake, Alaska, pelvic-reduced population (HUMP). The three different deletions in PAXB, BEPA, and HUMP overlap in a 488-bp region, each partially or completely removing the sequences found in the *Pel*-501-bp enhancer (Fig. 4A and figs. S4, S7, and S8).

To investigate whether a general mechanism and/or shared variants underlie repeated pelvic reduction in sticklebacks, we genotyped PAXB, BEPA, HUMP, and 10 additional pelvic-reduced populations from disparate geographic locations, as well as 21 pelvic-complete populations, using 149 single-nucleotide polymorphisms (SNPs) spanning 321 kb around the *Pitx1* locus (approximately 2-kb spacing) (fig. S8 and tables S1 and S3). Nine

of the 13 pelvic-reduced stickleback populations— but zero out of 21 pelvic-complete populations— showed consistent missing genotypes for multiple consecutive SNP markers located in and around the *Pel* enhancer (two-tailed *t* test, $P < 0.001$, df = 12.279) (Fig. 4A, fig. S8, and tables S4 and S5). 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-ligation 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
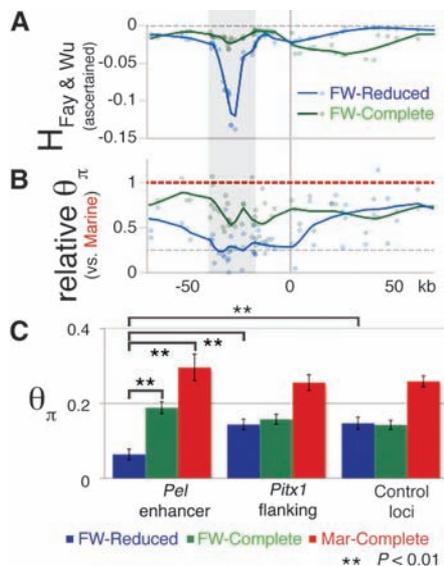
flexibility scores in the genome occur in the *Pitx1* region, suggesting that this region is exceptionally flexible and may be prone to deletion (Wilcoxon rank sum = 59,624, $P < 2 \times 10^{-6}$) (Fig. 4C).

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, $P < 0.01$) (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.
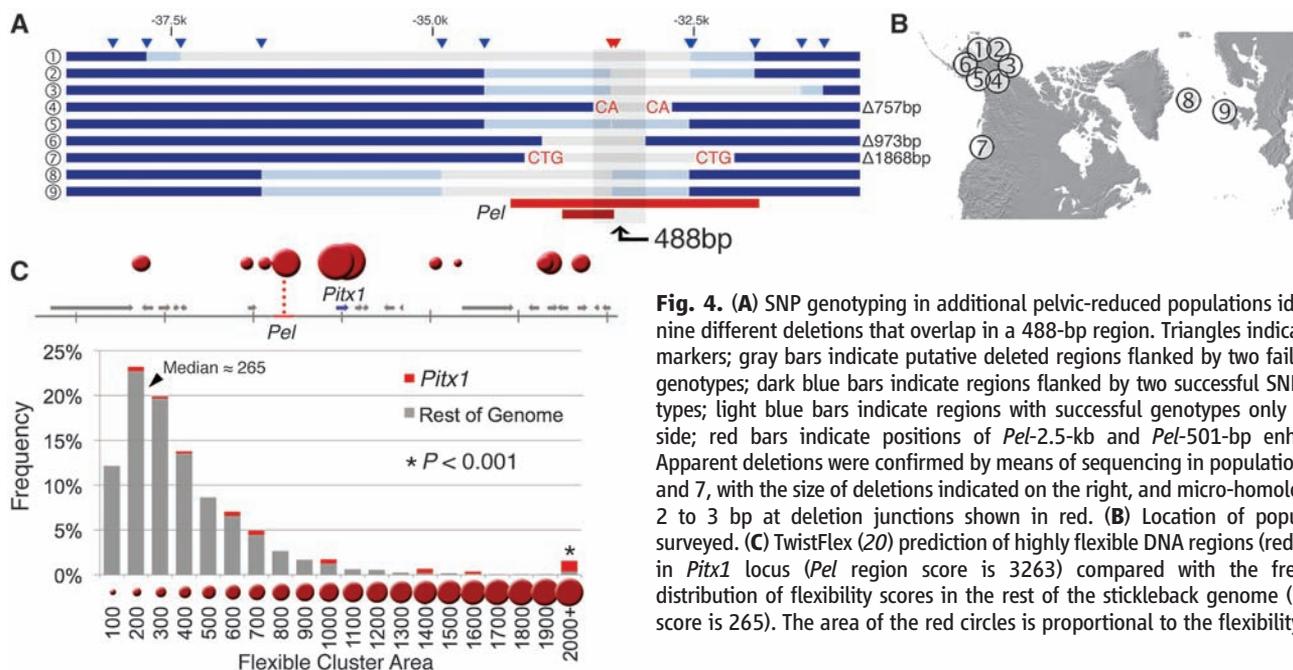
Fig. 4. (**A**) SNP genotyping in additional pelvic-reduced populations identifies nine different deletions that overlap in a 488-bp region. Triangles indicate SNP markers; gray bars indicate putative deleted regions flanked by two failed SNP genotypes; dark blue bars indicate regions flanked by two 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 (θ_π) statistics across the *Pitx1* region. Blue (freshwater pelvic-reduced) and green (freshwater pelvic-complete) data points and locally weighted scatterplot–smoothed ($\alpha = 0.2$) 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_π values are plotted relative to the grouped marine mean (per SNP) in order to control for variation in ascertainment between SNPs. **(C)** Heterozygosity (θ_π) 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).

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