Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks

Michael D. Shapiro[1]*, Melissa E. Marks[1]*, Catherine L. Peichel[2]*, Benjamin K. Blackman[1], Kirsten S. Nereng[1], Bjarni Jónsson[3], Dolph Schluter[4] & David M. Kingsley[1]

[1]*Department of Developmental Biology and HHMI, Stanford University School of Medicine, Stanford, California 94305-5329, USA*
[2]*Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA*
[3]*Institute of Freshwater Fisheries, Holar Agricultural College, IS-551 Saudarkrokur, Iceland*
[4]*Department of Zoology, University of British Columbia, 6270 University Blvd, Vancouver V6T 1Z4, Canada*

* These authors contributed equally to this work

Hindlimb loss has evolved repeatedly in many different animals by means of molecular mechanisms that are still unknown. To determine the number and type of genetic changes underlying pelvic reduction in natural populations, we carried out genetic crosses between threespine stickleback fish with complete or missing pelvic structures. Genome-wide linkage mapping shows that pelvic reduction is controlled by one major and four minor chromosome regions. *Pitx1* maps to the major chromosome region controlling most of the variation in pelvic size. Pelvic-reduced fish show the same left–right asymmetry seen in *Pitx1* knockout mice, but do not show changes in Pitx1 protein sequence. Instead, pelvic-reduced sticklebacks show site-specific regulatory changes in *Pitx1* expression, with reduced or absent expression in pelvic and caudal fin precursors. Regulatory mutations in major developmental control genes may provide a mechanism for generating rapid skeletal changes in natural populations, while preserving the essential roles of these genes in other processes.

Vertebrate limb structures exhibit extensive structural variation in animals adapted to different environments. In a phylogenetically diverse set of vertebrates—including lineages of reptiles, amphibians, marine mammals and fish—hindlimb structures are characteristically reduced or missing. The presence of rudimentary hindlimb structures in snakes and whales formed part of the early evidence indicating that these animals evolved from four-limbed ancestors through extensive modification of pre-existing skeletal structures[1,2]. Despite longstanding interest in the mechanisms that control hindlimb reduction during vertebrate evolution, the detailed number, location and type of genetic changes that underlie this process are still unknown[3–5].

Marine threespine sticklebacks (*Gasterosteus aculeatus*) have a prominent pelvic skeleton made up of bilateral pelvic spines that articulate in trochlear joints with an underlying pelvic girdle. The girdle covers part of the ventral surface and extends up the lateral side of the fish in an ascending branch that articulates with dermal armour plates (Fig. 1f). Previous studies suggest that pelvic structures protect sticklebacks against gape-limited, soft-mouthed predators by presenting a lacerating defensive structure, which increases the effective diameter of the fish and resists compressive forces during predator manipulation and chewing[6,7]. However, several freshwater stickleback populations have evolved complete or partial loss of the pelvic skeleton[8–12], perhaps in response to local absence of predatory fish, reduced levels of calcium ion availability, or predation by macroinvertebrates that typically capture sticklebacks by grasping the dorsal and pelvic spines[7,13–17]. The young geological age of the postglacial lakes containing pelvic-reduced sticklebacks, and the rapid tempo of pelvic changes seen in a high-resolution series of fossil sticklebacks[18], suggest that pelvic reduction can evolve in less than 10,000 generations in this system.

Genetic architecture of pelvic reduction

To examine the number and location of chromosome regions that control pelvic reduction in natural populations, we crossed a marine female showing robust development of pelvic structures to a freshwater male from the Paxton Lake benthic population[19]. As with the majority of the Paxton benthic population, this fish had no pelvic spines or underlying pelvic structures. A total of 375 adult F_2 progeny from a single pair of F_1 hybrid parents, both with complete pelves, were killed, measured for extent of development of several different pelvic features (Fig. 1f), and genotyped with a large set of previously described microsatellite markers[20].

Scoring pelvic reduction as a qualitative trait (normal pelvic structures versus any form of size reduction, loss or asymmetry) revealed a near 3:1 mendelian ratio of unaffected to affected fish (289 and 86 progeny, respectively). Mapping of this qualitative trait revealed strong linkage to markers on the distal end of linkage group 7 (log likelihood ratio of linkage (LOD) = 36.9). The same major chromosome region also controlled a substantial portion (13.5–43.7%) of the variance in every quantitative measure of pelvic size in the cross, including lengths of the pelvic spines and pelvic girdles, and heights of the ascending branches of the pelvis (Table 1 and Fig. 1). Several chromosome regions with smaller effects (minor modifier quantitative trait loci (QTL)) were also detected in the cross, each controlling between 5.6% and 11.1% of the variance in specific pelvic measurements, and mapping to several different linkage groups (Fig. 1b–e). The remaining variance may be due to additional minor loci with phenotypic effects that are too small to detect in a cross of this size, or to environmental and epigenetic factors. Each of the QTL detected in the current cross mapped to regions distinct from a previously reported QTL that modified pelvic spine length in fish from Priest Lake, British Columbia[20].

At each chromosome region that influenced pelvic morphology in the cross between marine and Paxton benthic sticklebacks, the net effect of freshwater alleles from the Paxton grandparent was to decrease the average size of pelvic structures (Table 1). Even fish with one marine and one Paxton benthic allele near a given QTL

showed a significant change in the mean size of pelvic structures compared with fish with two marine alleles at that locus (see mean phenotypes as a function of heterozygosity or homozygosity for Paxton benthic alleles near QTL on linkage groups 1, 4, 7 or 10 in Table 1). Increasing the total number of Paxton benthic alleles at the minor QTL on linkage groups 1, 2 and 4 produced larger combined effects than each minor modifier QTL individually (Fig. 1g, h). The magnitude of the phenotypic effect of the modifier QTL varied in fish that inherited only marine, marine and benthic, or only benthic alleles at the major QTL on linkage group 7 (see Supplementary Information). Both additive and epistatic interactions between loci are therefore likely to influence pelvic morphology. Rare, favourable, new alleles with semi-additive effects are expected to spread more rapidly in an evolving population than purely recessive alleles, when the alleles begin at low frequency[21]. In addition, traits controlled by a relatively small number of genes should evolve more quickly than traits that require the separate origin and fixation of new mutations at many different loci. Our results suggest that major morphological changes in the vertebrate hindlimb skeleton may occur through relatively few chromosome regions in natural populations. The heterozygous effects of Paxton benthic alleles, and the existence of a single major chromosome region controlling up to half of the phenotypic variance, may help to explain the rapid tempo of pelvic reduction in the stickleback system.

Isolation and mapping of candidate genes

Several genes have been described that are expressed specifically in hindlimbs but not forelimbs, or are required for normal hindlimb development in traditional vertebrate model systems. These genes include the transcription factors *Tbx4* (refs 22–24), *Pitx1* (refs 24–26) and *Pitx2* (ref. 27). To test whether variation in these genes may contribute to pelvic reduction in the Paxton benthic population, we isolated bacterial artificial chromosome (BAC) clones containing stickleback orthologues of each gene, sequenced portions of the clones to identify polymorphic genetic markers, and determined the segregation pattern of these markers in F_2 animals from the marine by Paxton benthic cross. The stickleback *Tbx4* and *Pitx2* markers mapped to linkage groups 1 and 4, respectively (Fig. 1b, d), clearly excluding them from the major chromosome region at the distal end of linkage group 7, which has the largest effect on pelvic reduction in the cross.

In contrast, a microsatellite marker located in the first intron of the *Pitx1* gene mapped to the distal end of linkage group 7, in a position indistinguishable from the major region controlling pelvic reduction (Fig. 1a). Addition of *Pitx1* to the linkage map substantially increased both the overall LOD score and the total variance explained by linkage group 7 for all the traits (Table 1), consistent with tight linkage between *Pitx1* and the pelvic reduction phenotype. A total of 70 F_2 progeny that showed complete bilateral absence of pelvic spines were identified from the mapping family and two additional families. Each of these fish was homozygous for Paxton benthic alleles at the *Pitx1* locus, showing that *Pitx1* does not recombine with the severe pelvic reduction phenotype in at least 140 chromosomes.

Left–right asymmetry in stickleback pelvic reduction

Pitx1 is a particularly intriguing candidate for the major locus controlling pelvic reduction in sticklebacks. During normal devel-

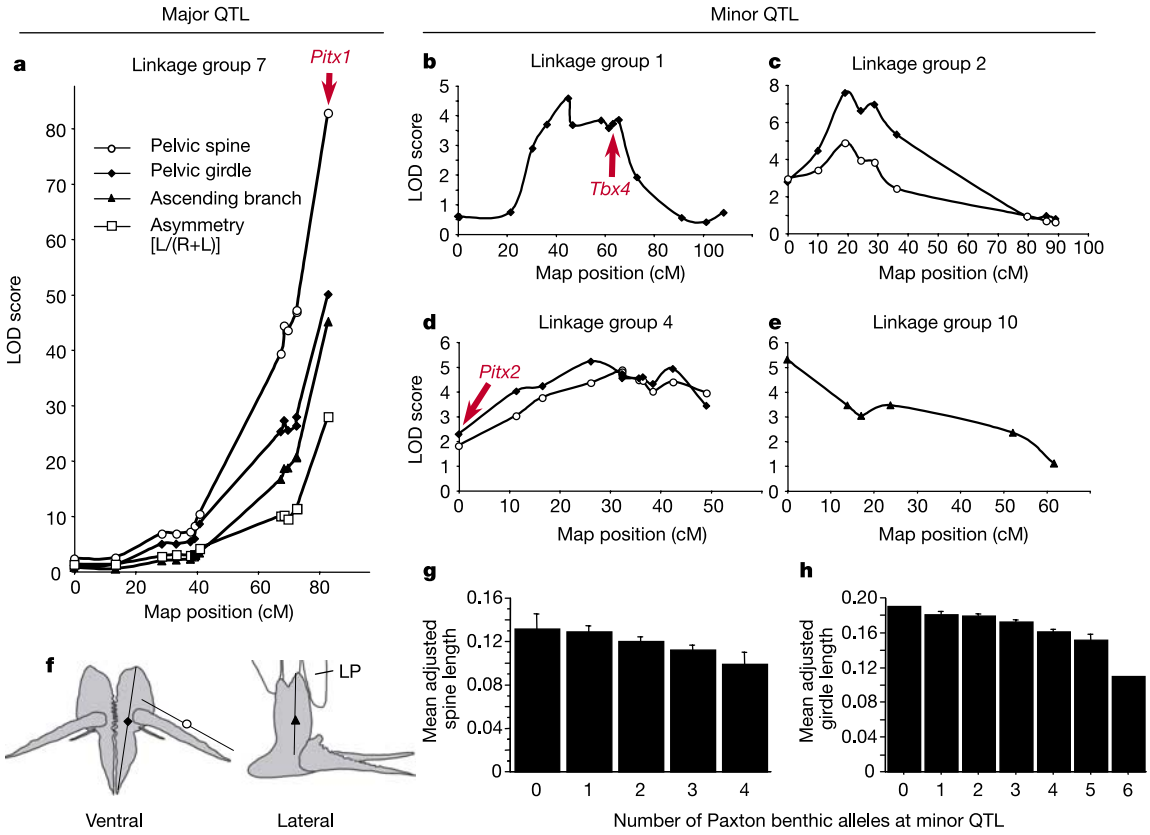

Figure 1 Genetic architecture of pelvic reduction in a cross between marine and Paxton benthic sticklebacks. **a**–**e**, LOD scores for different pelvic traits are graphed as a function of genetic distance (centimorgans; cM) along linkage groups[20] with significant QTL (LOD $\geq$ 4.5 (ref. 50)). (See Supplementary Information for markers.) **f**, Different quantitative measurements of pelvic structures. LP, lateral plates. **g**, **h**, Increasing substitution of Paxton benthic alleles at minor loci affecting spine length (**g**, linkage group 2, 4) or pelvic girdle length (**h**, linkage group 1, 2, 4) leads to a progressive reduction in the mean phenotypic values for each trait (multiple-way analysis of variance: $P < 0.03$, $P < 0.0001$, respectively). Error bars indicate standard error.

Table 1 **Location and magnitude of effect for major and minor pelvic reduction QTL**

Trait	LG	Marker	Map position (cM)	LOD	PVE (%)	Phenotype means		
						MM	MB	BB
Complete versus reduced pelvis	7	*Pitx1*	82.9	72.6	NA	NA	NA	NA
	7	*Stn82*	72.6	36.9	NA	NA	NA	NA
Pelvic spine length	7	*Pitx1*	82.9	82.8	65.3	0.148 ± 0.002	0.132 ± 0.001†	0.068 ± 0.004†§
	7	*Stn82*	72.6	45.5	43.7	0.144 ± 0.002	0.126 ± 0.002†	0.072 ± 0.005†§
	2	*Stn21*	19.1	4.9	7.6	0.115 ± 0.011	0.131 ± 0.005	0.098 ± 0.014‡
	4	*Gac4174*	32.4	4.9	5.8	0.134 ± 0.005	0.118 ± 0.003*	0.108 ± 0.004†
Pelvic girdle length	7	*Pitx1*	82.9	50.0	46.8	0.185 ± 0.001	0.178 ± 0.001*	0.140 ± 0.003†§
	7	*Stn82*	72.6	25.6	27.8	0.183 ± 0.003	0.174 ± 0.003*	0.144 ± 0.002†§
	1	*Stn7*	45.1	4.6	5.6	0.178 ± 0.002	0.170 ± 0.002*	0.160 ± 0.004†‡
	2	*Stn21*	19.1	7.6	11.1	0.169 ± 0.005	0.177 ± 0.004	0.147 ± 0.008*‡
	4	*Gac4174*	32.4	4.7	5.6	0.178 ± 0.002	0.171 ± 0.002	0.159 ± 0.004†‡
Ascending branch height	7	*Pitx1*	82.9	45.1	44.5	0.108 ± 0.001	0.106 ± 0.001	0.077 ± 0.002†§
	7	*Stn82*	72.6	19.8	22.2	0.107 ± 0.001	0.100 ± 0.002*	0.079 ± 0.003†§
	10	*Stn119*	0	5.3	6.6	0.105 ± 0.001	0.099 ± 0.002*	0.091 ± 0.002†‡
Asymmetry	7	*Pitx1*	82.9	28.0	31.5	0.504 ± 0.006	0.502 ± 0.001	0.701 ± 0.029†§
	7	*Stn82*	72.6	11.2	13.5	0.506 ± 0.006	0.525 ± 0.008	0.672 ± 0.030†§

Each significant QTL detected (LOD > 4.5) is shown with the corresponding linkage group (LG), maximum LOD score and percentage phenotypic variance explained (PVE). *Pitx1* is the most distal marker on linkage group 7, and substantially increased both the LOD and PVE scores compared with the previous most distal marker (*Stn82*). Mean length and height measures were calculated for progeny that inherited two marine (MM), one benthic and one marine (MB), or two benthic alleles (BB) at a marker closely linked to a given QTL (left side measure (mm)/standard body length (mm) ± standard error). Asymmetry is the ratio of left spine length to total combined spine length (left plus right). Statistical analysis was done using one-way analysis of variance. NA, not applicable.
*Significantly different from MM mean ($P < 0.03$).
†Significantly different from MM mean ($P < 0.0001$).
‡Significantly different from MB mean ($P < 0.003$).
§Significantly different from MB mean ($P < 0.0001$).

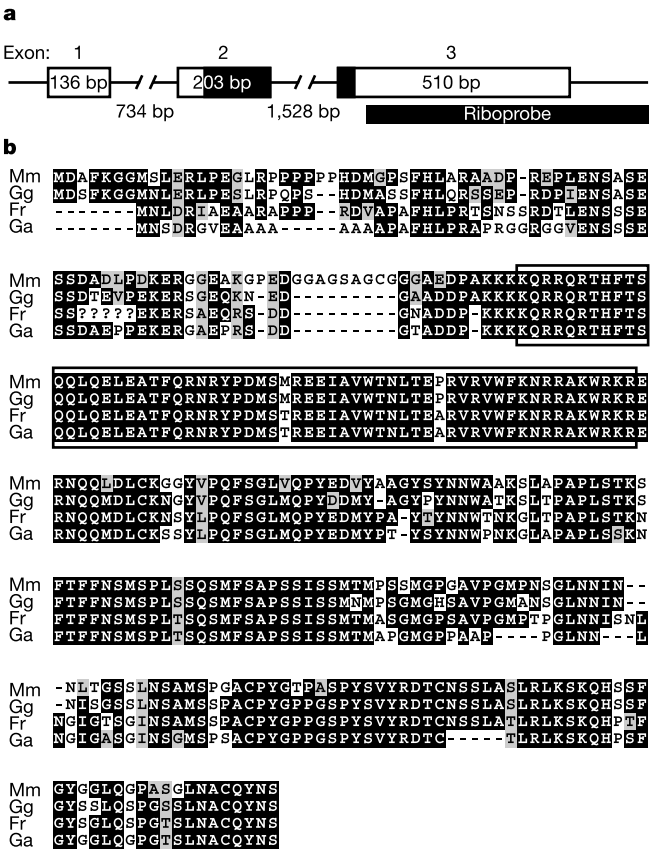

Figure 2 Structure and sequence of the stickleback *Pitx1* locus. **a**, The 849-bp *Pitx1* coding region comprises three exons. The homeodomain is indicated in black, and the sequence used for riboprobe is shown. **b**, Comparison of Pitx1 amino acid sequences for mouse (*Mus musculus*; Mm), chicken (*Gallus gallus*; Gg), pufferfish (*Fugu rubripes*; Fr) and threespine stickleback (*G. aculeatus*; Ga). Identical amino acids are shaded black whereas conservative substitutions are shaded grey. The homeodomain is boxed. Sequence ambiguities in the predicted pufferfish sequence are denoted by a question mark. Amino acid sequence and splicing of the *Pitx1* transcript is identical in marine and Paxton benthic sticklebacks with complete and reduced pelves.

opment in the mouse, this gene is highly expressed in hindlimbs but not in forelimbs, giving rise to the alternative descriptive name backfoot[28]. Null mutations in the gene lead to selective reduction of hindlimbs but not forelimbs. Pelvic limb reduction in *Pitx1* mouse mutants also shows strong directional asymmetry, with greater reduction on the right than on the left side[25,27]. This effect is probably due to partial functional compensation by the closely related gene *Pitx2* (ref. 27), which is expressed preferentially on the left side during development, a laterality that is highly conserved in mammals, frogs and fish[29]. To test for similar morphological directional asymmetry in the cross between marine and Paxton benthic sticklebacks, we measured lengths of both left and right pelvic structures, and scored asymmetry as the ratio of the length of the spine on the left to the combined length of both sides. We observed strong directional asymmetry in the F_2 progeny, with longer spines on the left than right side in 78% of animals with asymmetric development (97 out of 124), and a mean ratio of left to total spine length of 0.719 ($P < 0.0001$, one-sided *t*-test). Mapping pelvic asymmetry as a quantitative trait showed strong linkage to the *Pitx1* locus on the distal end of linkage group 7 (LOD = 31.5, Table 1 and Fig. 1a). Directional asymmetry of pelvic reduction in sticklebacks may thus be a naturally occurring phenocopy of the hindlimb-specific directional asymmetry previously seen in laboratory-generated *Pitx1* knockout mice.

Pitx1 sequence comparison in marine and benthic fish

To test whether populations with pelvic reduction have changes in the coding regions of *Pitx1*, we determined the exon/intron structure of the gene (Fig. 2a), and sequenced the entire coding region and exon–intron junctions in both marine and Paxton benthic individuals. The three exons of the stickleback *Pitx1* gene encode a 283-amino-acid protein that shows extensive sequence identity to Pitx1 sequences previously reported from other fish, birds and mammals (Fig. 2b). No coding region mutations were found that would alter the amino acid sequence of the gene product in the pelvic-reduced Paxton benthic population relative to the marine population. Reverse-transcriptase-mediated polymerase chain reaction (RT–PCR) and sequencing studies confirmed that both marine and Paxton benthic fish correctly assembled the three exons into an intact, spliced *Pitx1* transcript (data not shown).

Altered *Pitx1* gene expression in pelvic-reduced fish

To test for possible regulatory changes in the *Pitx1* locus, we

examined the spatial patterns of expression of *Pitx1* during normal development of both marine and Paxton benthic larvae. In marine fish, *Pitx1* was clearly expressed in bilateral patches of mesenchymal cells just posterolateral to the anterior margin of the ventral median fin, at the site where the pelvic fin bud normally develops (Fig. 3). In addition, *Pitx1* expression was detected at several other sites including the developing thymus, olfactory pits, sensory neuromasts on the head, trunk and tail, and the ventral portion of the developing caudal fin (Fig. 4 and data not shown). Larvae from the Paxton benthic population also showed abundant *Pitx1* expression in the developing thymus, olfactory pits and neuromasts of the head, trunk and tail (Fig. 4 and data not shown). However, no *Pitx1* expression was detectable in the prospective pelvic region (Fig. 3) of Paxton benthic larvae, and expression in the developing caudal fin was also much weaker than in the marine population (Fig. 4). Note that the caudal fin is present in both marine and Paxton benthic larvae, and no significant changes in caudal fin ray number are seen in F_2 progeny that inherit different alleles at the *Pitx1* locus (data not shown). The altered fin expression pattern of *Pitx1* thus cannot be due solely to absence of some structures in Paxton benthic fish. Furthermore, lack of expression in the prospective pelvic region was not due to a developmental delay or difference in timing, as marine larvae continued to show robust expression of *Pitx1* in the pelvic region for several days, whereas no expression was ever detected in the corresponding region of Paxton benthic larvae (Fig. 3 and data not shown). Paxton benthic fish thus show an altered pattern of *Pitx1* expression in some tissues but not others, consistent with a regulatory mutation that disrupts expression in both the prospective pelvic region and caudal fin.

Parallel evolution of pelvic reduction

Pelvic reduction has evolved multiple times in widely separated lakes throughout the Northern Hemisphere[8–12]. To test whether the genetic basis of pelvic loss is similar in independent locations, we established a complementation cross between pelvic-reduced fish from Paxton Lake, British Columbia, and Lake Vífilsstadavatn, Iceland. The Vífilsstadavatn population showed pronounced pelvic reduction, with greater reduction on the right than left side (mean pelvic scores using a standard 0 (all pelvic elements absent) to 4 (all elements present) scoring system[16]: 2.78 (left) and 2.12 (right); distribution of scores, 0 = 1.5%, 1 = 12.6%, 2 = 18.6%, 3 = 41.2%, 4 = 26.1% (left) and 0 = 11.1%, 1 = 24.7%, 2 = 16.7%, 3 = 34.8%, 4 = 12.6% (right), $n = 199$). The individual fish chosen for the complementation cross had pelvic scores of 0 (Paxton benthic) and 1 (Vífilsstadavatn) on both sides. All 39 F_1 hybrid fish from these parents also showed highly reduced pelvic development: 14 fish had no trace of a pelvic skeleton, 9 had bilateral ovoid rudiments, 15 had a small pelvic rudiment on the left side only and 1 had a small rudiment on the right side only (mean pelvic scores: 0.61 (left), 0.26 (right)). Therefore, the pelvic reduction alleles in these two populations failed to complement and restore normal pelvic morphology. In contrast, a control cross between a Vífilsstadavatn pelvic-reduced fish (pelvic score of 1 on each side) and a Little Campbell River marine fish with a robust pelvis (pelvic score of 4 on each side) produced F_1 hybrid fish that all showed strong bilateral development of both pelvic spines and girdles

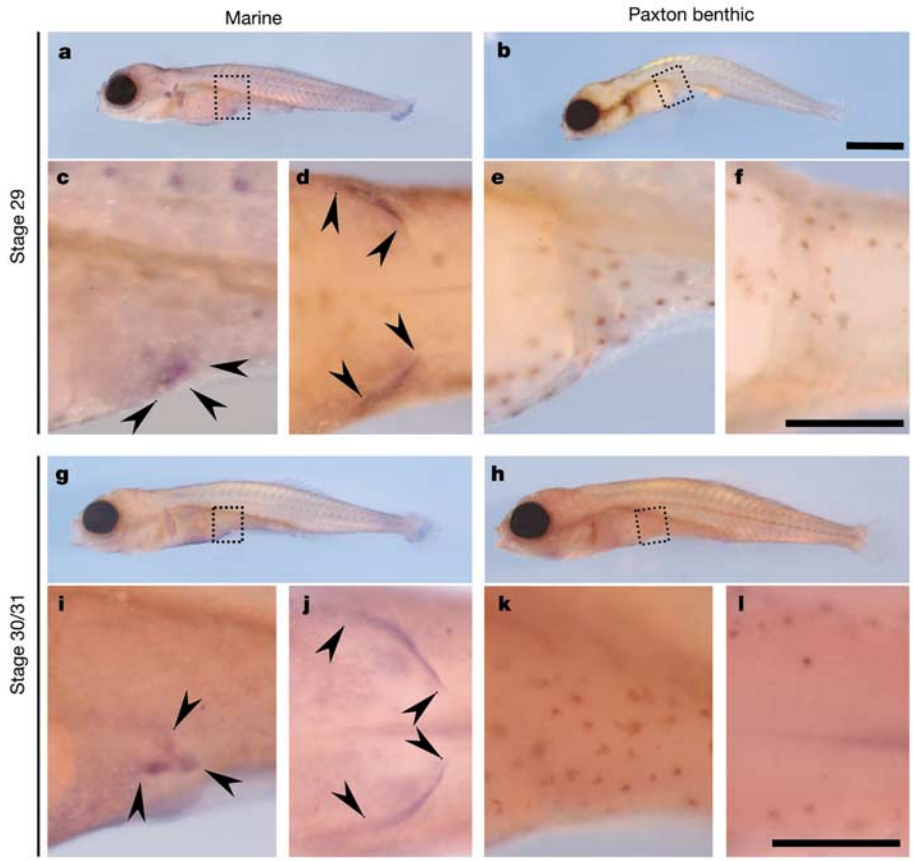

Figure 3 *Pitx1* is expressed in the prospective pelvic region of marine but not Paxton benthic sticklebacks. **a**, Whole-mount *in situ* hybridization shows *Pitx1* expression in the prospective pelvic region of stage 29 marine larvae. Arrowheads mark limits of expression in enlarged lateral (**c**) and ventral (**d**) views. **b**, **e**, **f**, Conversely, no *Pitx1* expression was detected in the corresponding regions of Paxton benthic larvae. Similar results are seen at later developmental stages (stage 30/31) of marine (**g**, **i**, **j**) and Paxton benthic (**h**, **k**, **l**) larvae. Scale bars: 1 mm (**a**, **b**, **g**, **h**); 0.25 mm (**c**–**f**, **i**–**l**).

(mean pelvic scores: 4.0 (left), 4.0 (right), $n = 41$). These findings indicate that failure of pelvic development in the cross between Paxton and Vífilsstadavatn fish was not due to dominant genetic changes in the Vífilsstadavatn population. The directional asymmetry in the Icelandic population, and the failure to restore pelvic development in crosses with Paxton benthic fish, suggest that pelvic reduction in these two distinct Pacific and Atlantic basin populations results from defects in similar genes.

Discussion

We have identified a major chromosome region controlling loss of pelvic structures in a natural population of sticklebacks. The absence of recombination between *Pitx1* and the major pelvic-reduction phenotype, the strong directional asymmetry seen in pelvic-reduced fish, and the altered pattern of *Pitx1* expression seen during normal development all suggest that *cis*-acting regulatory mutations in *Pitx1* are a major cause of pelvic reduction in this rapidly evolving system (Fig. 5). Notably, complete inactivation of the *Pitx1* gene in mice leads to severe craniofacial abnormalities, pituitary defects and neonatal lethality[25,26]. Similarly, null mutations in other known limb-identity genes such as *Tbx4* (ref. 30) and *Tbx5* (refs 31, 32) also cause embryonic lethality due to the pleiotropic roles of these genes in development of other tissues. In contrast, Paxton benthic fish show no alterations in *Pitx1* coding sequences, and changes in gene regulation that disrupt expression only at specific sites in developing larvae. Regulatory mutations in key developmental control genes may provide a general mechanism to selectively alter expression in specific structures and yet preserve expression at other sites required for viability[33–35]. Similar site-specific regulatory mutations are thought to underlie microevolutionary changes in trichromes, larval hairs, pigmentation and wing eyespot size of fruitflies and butterflies[36–39], and muscle mass variation in pigs[40]. Our study suggests that regulatory mutations in key developmental control genes may also be responsible for major and rapid morphological changes in limb and fin structures during vertebrate evolution.

In this and many other cases where evolutionary changes have been traced to regulatory alterations in major developmental control genes, the actual DNA sequences responsible for tissue-specific expression differences are still unknown[36–39]. Regulatory mutations are much more difficult to identify at the molecular level than coding region mutations, and could be located at large distances either 5′ or 3′ to the gene of interest. Comparative analysis of mouse and human genome sequences shows that the *Pitx1* gene is flanked by large regions of potential regulatory sequences, including more than 300 kilobases of flanking DNA that is highly enriched in conserved non-coding sequences[41]. These sequences probably include multiple regulatory elements controlling *Pitx1* gene expression in different tissues (Fig. 5c). A major goal of future research will be to identify particular DNA sequences that normally control expression of *Pitx1* in developing hindlimbs (fins), and to compare the structure and function of these regions in marine and Paxton benthic fish.

Are mutations in *Pitx1* likely to underlie other examples of naturally occurring pelvic reduction? Pelvic-reduced populations have evolved repeatedly from marine ancestors in many different locations. The complementation cross between Paxton and Icelandic fish suggests that pelvic reduction has occurred by similar genetic

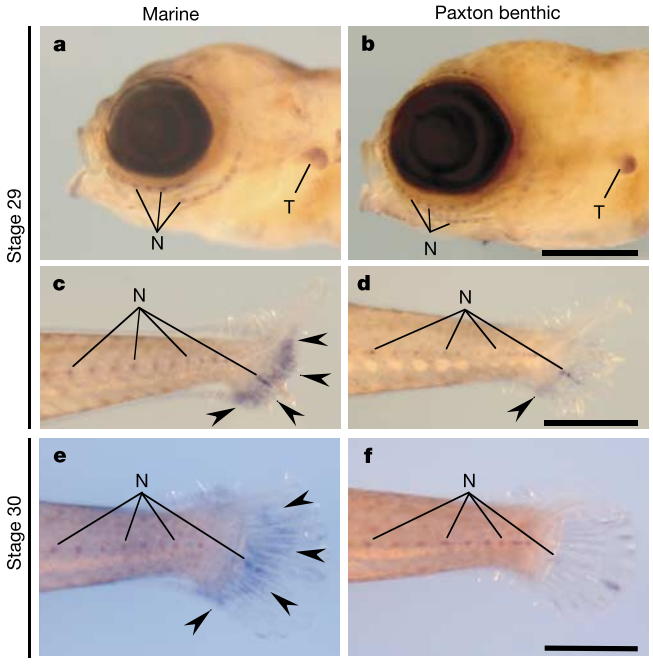

Figure 4 Site-specific regulatory changes of *Pitx1* expression. **a**–**f**, *Pitx1* is expressed at similar levels in several non-fin tissues of stage 29 and stage 30 marine (**a**, **c**, **e**) and Paxton benthic (**b**, **d**, **f**) larvae, including thymus (T) and cranial and trunk neuromasts (N). At stage 29, however, *Pitx1* expression is considerably higher in the ventral caudal fins (arrowheads) of marine (**c**) than Paxton benthic larvae (**d**). At stage 30, caudal fins undergo normal dorsal rotation in both marine (**e**) and Paxton benthic (**f**) populations, but only the marine population shows expression of *Pitx1* in the caudal fin region. Scale bars: 0.5 mm.

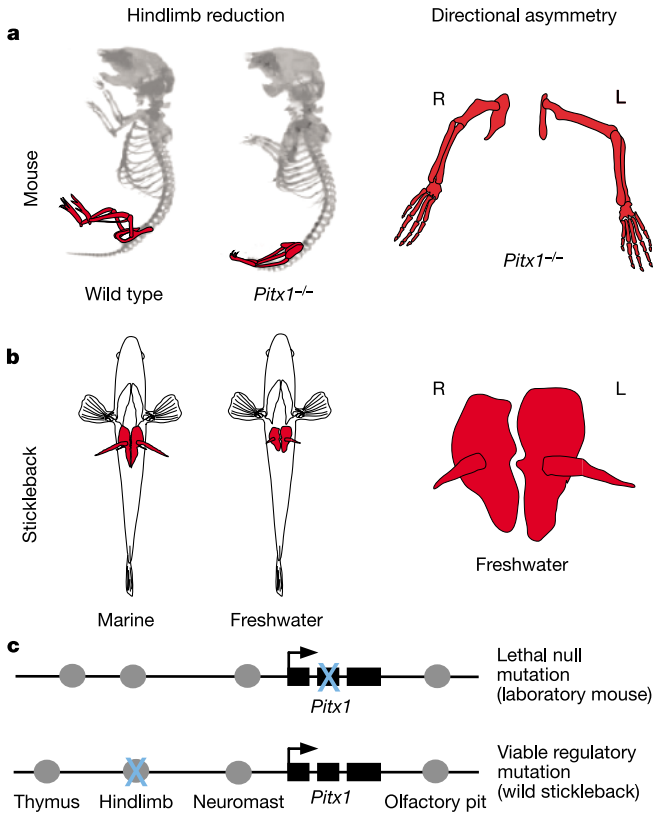

Figure 5 Comparison of pelvic reduction in laboratory and natural populations. **a**, Inactivation of *Pitx1* in laboratory mice causes a marked decrease in size of hindlimbs but not forelimbs (modified from refs 25, 27), and greater reduction on the right than left side. **b**, Sticklebacks show similar *Pitx1*-linked selective reduction and asymmetry in hindlimbs. **c**, Null mutations in *Pitx1* in mice disrupt multiple functions and lead to neonatal lethality. In contrast, regulatory mutations in modular *cis*-acting regulatory elements of sticklebacks could produce major morphological changes in particular body regions without disrupting functions in other tissues.

mechanisms in populations located more than 5,700 km apart. Recent experiments also have shown that both *Pitx1* and *Tbx4* fail to be expressed in the prospective pelvic region of pelvic-reduced fish in Scotland[42]. Many other populations show the same left–right asymmetry that is a characteristic feature of *Pitx1*-linked pelvic reduction in mice and Paxton benthic fish, including multiple populations of *Gasterosteus aculeatus*[13,42,43], a 10-million-year-old fossil sample of *G. doryssus*[18] and pelvic-reduced populations from the distantly related stickleback genera *Culea*[44] and *Pungitius*[45]. Mutations in or closely linked to the *Pitx1* locus may contribute to many other examples of evolutionary reduction of pelvic structures in natural populations, a possibility that can now be tested by further genetic studies and direct analysis of *Pitx1* structure and regulation in multiple populations, species and genera. □

Methods

Fish crosses and husbandry

A wild-caught marine female stickleback from Onnechikappu stream (east coast of Hokiiado Island, Japan) was crossed to a wild-caught benthic male from Paxton Lake (British Columbia). Hybrid progeny were raised to maturity in 30-gallon aquaria. F_1 hybrids were mated in pairs, and 2,600 F_2 progeny were raised to a standard length of >30 mm. A total of 375 full siblings from a single F_2 family were used for detailed phenotype and genotype studies. Nineteen fish from this family, and 51 fish from two additional F_2 families, showed complete bilateral absence of pelvic spines and were used for finer recombination mapping of *Pitx1*.

Wild-caught Gjogur marine (Iceland) and Paxton Lake sticklebacks were spawned in the laboratory for *in situ* hybridization studies. Progeny were raised in 15-gallon aquaria at 18 °C.

Complementation crosses were set up between wild-caught, pelvic-reduced fish from Lake Vífilsstadavatn (Gardabaer, Iceland) and reduced- or full-pelvis fish from Paxton Lake or Little Campbell River (British Columbia). Crosses were raised in 30-gallon aquaria at 18 °C and scored for pelvic phenotypes after 4 months.

Morphological analysis and QTL mapping

Fish were fixed and measured as described[20]. Pelvic spines were measured from the distal tip to the point of articulation with the pelvis, pelvic girdles from the anterior to posterior tip, and ascending branch from the dorsal tip to the point of articulation with the spine. Asymmetry in the pelvis was analysed as the ratio of left spine length to total combined spine length. Phenotypic measures were analysed with MapQTL 3.0 (ref. 46) as described[20], using either raw pelvic measurements, measurements scaled to standard lengths of fish, or residuals of a regression of pelvic measurements on standard body length. Each method led to identification of similar major and minor chromosome regions.

BAC library screening, sequencing and genotyping

A stickleback BAC library (CHORI-213; Children's Hospital Oakland Research Institute) prepared from marine fish (Salmon River, British Columbia) with robust pelvic structures was screened on high-density filters using radiolabelled overgo oligonucleotide probes[47] designed to conserved *Pitx1*, *Pitx2* and *Tbx4* coding sequences: *Pitx1* screen, 5′-TTTGATCGGCTCCCGTCA-3′ and 5′-GTGCCAGTACAACAGCTGACGGGA-3′, 5′-CGCTCAGACTCAAGTCCAAACAG-3′ and 5′-GCCGAAGCTCGGGTGCTGTT TGG-3′; *Pitx1*/*Pitx2* combined screen, 5′-CTGTCCACCAAGAACTTCACTTTC-3′ and 5′-GGCTCATGGAGTTGAAGAAAGTGA-3′, 5′-CTTCAACTCCATGAGCCCTCT TAC-3′ and 5′-GAACATGGACTGAGATGTAAGAGG-3′, 5′-CTTCACTTTCTTCAAC TCCATGAG C-3′ and 5′-GACTGAGATGTAAGAGGGCTCATGG-3′, 5′-TGGAGTT GAAGAAAGTGAA-3′ and 5′-CCCGCTGTCCACCAAGAACTTCACTTT-3′; *Tbx4* screen, 5′-TGGATCCAAGAACACGGCCTACTG-3′ and 5′-GTGGAAGACATGGGTACA GTAGGC-3′, 5′-ATCCAAGAACACGGCCTACTGTA-3′ and 5′-CGTGGAAGACAT GGGTACAGTAG-3′. Positive BAC clones were secondarily screened using PCR with gene-specific primers: *Pitx1* and *Pitx2*, 5′-AGCCGTACGAAGACATGTACC-3′ and 5′-CATGGTCATGGAGGAGATGG-3′; *Tbx4*, 5′-CCCATTTCGAACCACTTCC-3′ and 5′-AGTAATGCTCCTCCGAGACC-3′. Clones were end-sequenced using T7 and SP6 primers on an ABI377 or ABI3730xl automatic sequencer, followed by complete (*Pitx1*) or partial (*Pitx2*, *Tbx4*) sequencing of coding regions (GenBank accession numbers AY517634–AY517636). Genetic mapping was carried out with PCR primers that flanked a trinucleotide repeat in intron 1 of *Pitx1* (set 1, 5′-TCGACGAGACTCATCTCACG-3′ and 5′-AATGTTTGCTCGGTGTTTCC-3′; set 2, 5′-AAACCGGAGAAGGTTAAACG-3′ and 5′-GACTGCTCCGTTTGATGAGG-3′), a four-nucleotide polymorphism in the T7 end of *Pitx2* BAC clone 59-O19 (5′-GTCTCTGTCTCAGGCTTGGC-3′ and 5′-AAGGCTGT GACTGTAAAAAGGG-3′; GenBank AY517637), and a dinucleotide repeat in the T7 end of *Tbx4* BAC clone 143-J5 (5′-CTGCTCTTCTTTTTGTCACCC-3′ and 5′-TCTCCTCT ACCACATGAAGGG-3′; GenBank AY517638). Each marker was amplified from genomic DNA of F_2 progeny as described[20].

Pitx1 sequence comparisons

PCR primers flanking exons 1, 2 and 3 were used to amplify *Pitx1* coding regions from Paxton Lake fish (Exon 1, 5′-GAGGGCAAATGTTTACTCAGC-3′ and 5′-CATTCTTCCC TGACAGACTGC-3′; Exon 2, 5′-GTGCTGGAAGAACTTCAGGG-3′ and 5′-CAGCGT GACTAATAGAACGGG-3′; Exon 3, 5′-ACCTTTGTTGGTAAATCCGC-3′ and 5′-ACACAAAACCCGCATAATCC-3′). Products were cloned into pCR2.1-TOPO (Invitrogen), sequenced, and aligned using Sequencher 4.1 software.

RT–PCR

Complementary DNA was synthesized (RETROScript, Ambion) using total RNA from marine and Paxton benthic fish. A 756-base-pair (bp) fragment of the *Pitx1* transcript that included the 3′ portion of exon 1, all of exons 2 and 3, and 6 bp of 3′ untranslated region was amplified and sequenced as above (primers 5′-CCGCGTTTCACTTACCGAGAG-3′ and 5′-CTCCCGTCAGCTGTTGTACTG-3′).

In situ hybridization

A 656-bp fragment of *Pitx1* was amplified by PCR from CHORI-213 BAC clone 164-F21 using primers 5′-agaatggatccCCTGTGCAAGAGCAGCTACC-3′ and 5′-actattctagaGCGCCTTCATCATAAAAAGC-3′, where lower-case letters indicate engineered restriction sites, and upper-case letters indicate *Pitx1*-specific sequence. The amplified fragment was cloned into the pCR4-TOPO cloning vector (Invitrogen), and riboprobe synthesis was performed as described[48].

For whole-mount *in situ* hybridization, marine and Paxton benthic larvae were staged under a dissecting microscope using morphology of the head, and caudal, dorsal and anal fins[49], fixed overnight at 4 °C in 4% paraformaldehyde in phosphate buffered saline (PBS; Gibco), and stored at −20 °C in methanol. Rates of larval development were slightly variable within and among clutches. Stage 29 marine larvae were fixed at 14 days post-fertilization (d.p.f.), Paxton benthics at 18 d.p.f.; stage 30/31 marine larvae at 20 d.p.f., Paxton benthics at 29 d.p.f. *In situ* hybridizations were performed essentially as described[48]. After signal development in BCIP/NBT (SigmaFast tablets, Sigma), the alkaline phosphatase reaction was halted by washing in 100 mM glycine in PBT (PBS plus 0.1% Tween-20), followed by dehydration with two 5-min washes in 100% ethanol, and re-hydration into PBT. Larvae were post-fixed in 4% paraformaldehyde and photographed in PBT.

Received 13 November 2003; accepted 30 January 2004; doi:10.1038/nature02415.

1. Darwin, C. *The Origin of Species* 450–456 (John Murray, London, 1859).
2. Struthers, J. On the bones, articulations, and muscles of the rudimentary hind-limb of the Greenland right-whale (*Balaena mysticetus*). *J. Anat. Phys.* **15,** 141–176, 302–321 (1881).
3. Bejder, L. & Hall, B. K. Limbs in whales and limblessness in other vertebrates: mechanisms of evolutionary and developmental transformation and loss. *Evol. Dev.* **4,** 445–458 (2002).
4. Lande, R. Evolutionary mechanisms of limb loss in tetrapods. *Evolution* **32,** 73–92 (1978).
5. Cohn, M. J. & Tickle, C. Developmental basis of limblessness and axial patterning in snakes. *Nature* **399,** 474–479 (1999).
6. Hoogland, R. D., Morris, D. & Tinbergen, N. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defense against predators (*Perca* and *Esox*). *Behaviour* **10,** 205–230 (1957).
7. Reimchen, T. E. Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). *Evolution* **37,** 931–946 (1983).
8. Bell, M. A. Reduction and loss of the pelvic girdle in *Gasterosteus* (Pisces): a case of parallel evolution. *Nat. Hist. Mus. LA Contrib. Sci.* **257,** 1–36 (1974).
9. Moodie, G. E. E. & Reimchen, T. Phenetic variation and habitat differences in *Gasterosteus* populations of the Queen Charlotte Islands. *Syst. Zool.* **25,** 49–61 (1976).
10. Campbell, R. N. & Williamson, R. B. The fishes of inland waters in the Outer Hebrides. *Proc. R. Soc. Edinb.* **77B,** 377–393 (1979).
11. Edge, T. A. & Coad, B. W. Reduction of the pelvic skeleton in the three-spined stickleback *Gasterosteus aculeatus* in 2 lakes of Quebec Canada. *Can. Field-Nat.* **97,** 334–336 (1983).
12. Bell, M. A. Interacting evolutionary constraints in pelvic reduction of threespine sticklebacks, *Gasterosteus aculeatus* (Pisces, Gasterosteidae). *Biol. J. Linn. Soc.* **31,** 347–382 (1987).
13. Reimchen, T. E. Spine deficiency and polymorphism in a population of *Gasterosteus aculeatus*—an adaptation to predators. *Can. J. Zool.* **58,** 1232–1244 (1980).
14. Reist, J. D. Predation upon pelvic phenotypes of brook stickleback, *Culaea inconstans*, by selected invertebrates. *Can. J. Zool.* **58,** 1253–1258 (1980).
15. Giles, N. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer-Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. *J. Zool.* **199,** 535–544 (1983).
16. Bell, M. A., Orti, G., Walker, J. A. & Koenings, J. P. Evolution of pelvic reduction in threespine stickleback fish—a test of competing hypotheses. *Evolution* **47,** 906–914 (1993).
17. Ziuganov, V. V. & Zotin, A. A. Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. *Behaviour* **132,** 1095–1105 (1995).
18. Bell, M. A., Baumgartner, J. V. & Olson, E. C. Patterns of temporal change in single morphological characters of a Miocene stickleback fish. *Paleobiology* **11,** 258–271 (1985).
19. McPhail, J. D. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): evidence for a species pair in Paxton Lake, Texada Island, British Columbia. *Can. J. Zool.* **70,** 361–369 (1992).
20. Peichel, C. L. *et al.* The genetic architecture of divergence between threespine stickleback species. *Nature* **414,** 901–905 (2001).
21. Crow, J. F. & Kimura, M. *An Introduction to Population Genetics Theory* 182–183 (Burgess, Minneapolis, 1970).
22. Gibson-Brown, J. J. *et al.* Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev.* **56,** 93–101 (1996).
23. Logan, M., Simon, H. G. & Tabin, C. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* **125,** 2825–2835 (1998).
24. Logan, M. & Tabin, C. J. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. *Science* **283,** 1736–1739 (1999).
25. Lanctôt, C., Moreau, A., Chamberland, M., Tremblay, M. L. & Drouin, J. Hindlimb patterning and mandible development require the Ptx1 gene. *Development* **126,** 1805–1810 (1999).
26. Szeto, D. P. *et al.* Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* **13,** 484–494 (1999).
27. Marcil, A., Dumontier, E., Chamberland, M., Camper, S. A. & Drouin, J. Pitx1 and Pitx2 are required for development of hindlimb buds. *Development* **130,** 45–55 (2003).

28. Shang, J., Luo, Y. & Clayton, D. A. Backfoot is a novel homeobox gene expressed in the mesenchyme of developing hind limb. *Dev. Dyn.* **209,** 242–253 (1997).
29. Campione, M. *et al.* The homeobox gene Pitx2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. *Development* **126,** 1225–1234 (1999).
30. Naiche, L. A. & Papaioannou, V. E. Loss of Tbx4 blocks hindlimb development and affects vascularization and fusion of the allantois. *Development* **130,** 2681–2693 (2003).
31. Bruneau, B. G. *et al.* A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106,** 709–721 (2001).
32. Garrity, D. M., Childs, S. & Fishman, M. C. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129,** 4635–4645 (2002).
33. Carroll, S. B. Endless forms: the evolution of gene regulation and morphological diversity. *Cell* **101,** 577–580 (2000).
34. Stern, D. L. Evolutionary developmental biology and the problem of variation. *Evolution* **54,** 1079–1091 (2000).
35. Tautz, D. Evolution of transcriptional regulation. *Curr. Opin. Genet. Dev.* **10,** 575–579 (2000).
36. Stern, D. L. A role of Ultrabithorax in morphological differences between *Drosophila* species. *Nature* **396,** 463–466 (1998).
37. Sucena, E. & Stern, D. L. Divergence of larval morphology between *Drosophila sechellia* and its sibling species caused by cis-regulatory evolution of ovo/shaven-baby. *Proc. Natl Acad. Sci. USA* **97,** 4530–4534 (2000).
38. Kopp, A., Duncan, I., Godt, D. & Carroll, S. B. Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408,** 553–559 (2000).
39. Beldade, P., Brakefield, P. M. & Long, A. D. Contribution of Distal-less to quantitative variation in butterfly eyespots. *Nature* **415,** 315–318 (2002).
40. van Laere, A. S. *et al.* A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. *Nature* **425,** 832–836 (2003).
41. Schwartz, S. *et al.* Human-mouse alignments with BLASTZ. *Genome Res.* **13,** 103–107 (2003).
42. Cole, N. J., Tanaka, M., Prescott, A. & Tickle, C. Expression of limb initiation genes and clues to the morphological diversification of threespine stickleback. *Curr. Biol.* **13,** R951–R952 (2003).
43. Bell, M. A., Francis, R. C. & Havens, A. C. Pelvic reduction and its directional asymmetry in threespine sticklebacks from the Cook Inlet region, Alaska. *Copeia* **1985,** 437–444 (1985).
44. Nelson, J. S. Evidence of a genetic basis for absence of pelvic skeleton in brook stickleback, *Culaea inconstans*, and notes on geographical distribution and origin of loss. *J. Fish. Res. Board Can.* **34,** 1314–1320 (1977).
45. Nelson, J. S. Absence of pelvic complex in ninespine sticklebacks, *Pungitius pungitius*, collected in Ireland and Wood Buffalo National Park region, Canada, with notes on meristic variation. *Copeia* **1971,** 707–717 (1971).
46. van Ooijen, J. W. & Maliepard, C. *MapQTL Version 3.0: Software for the Calculation of QTL Positions and Genetic Maps* (Centre for Plant Breeding and Reproductive Research, Wageningen, 1996).
47. Ross, M. T., LaBrie, S., McPherson, J. & Stanton, V. P. in *Current Protocols in Human Genetics* (ed. Dracopoli, N.) (Wiley, New York, 1999).
48. Wilkinson, D. G. in *Essential Developmental Biology: A Practical Approach* (eds Stern, C. D. & Holland, P. W. H.) 257–274 (IRL Press, Oxford, 1993).
49. Swarup, H. Stages in the development of the stickleback *Gasterosteus aculeatus* (L.). *J. Embryol. Exp. Morphol.* **6,** 373–383 (1958).
50. Van Ooijen, J. W. LOD significance thresholds for QTL analysis in experimental populations of diploid species. *Heredity* **83,** 613–624 (1999).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank S. Mori for providing marine sticklebacks; E. Elfarsdóttir, G. I. Gudbrandsson, L. E. Jónsdóttir, S. Ingólfsdóttir and K. Einarsdóttir for collecting Icelandic sticklebacks and scoring the parental population from Vífilsstadavatn; and F. Chan and members of the Kingsley laboratory for discussions. This work was supported in part by grants from the National Institutes of Health (to D.M.K.), the Natural Sciences and Engineering Research Council of Canada and the Canada Foundation for Innovation (to D.S.), and the City of Gardabaer (to B.J.); a Helen Hay Whitney Foundation postdoctoral fellowship (to M.D.S.); and a Howard Hughes Medical Institute predoctoral fellowship (to M.E.M). D.S. is a Canada Research Chair, and D.M.K. is an Associate Investigator of the Howard Hughes Medical Institute.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.M.K. (kingsley@cmgm.stanford.edu). The sequences described in this paper have been deposited in GenBank under accession numbers AY517634–AY517638.