

Parallel Evolution of *Pitx1* Underlies Pelvic Reduction in Scottish Threespine Stickleback (*Gasterosteus aculeatus*)

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Abstract

Little is known about the genetic and molecular mechanisms that underlie adaptive phenotypic variation in natural populations or whether similar genetic and molecular mechanisms are utilized when similar adaptive phenotypes arise in independent populations. The threespine stickleback (*Gasterosteus aculeatus*) is a good model system to investigate these questions because these fish display a large amount of adaptive phenotypic variation, and similar adaptive phenotypes have arisen in multiple, independent stickleback populations. A particularly striking pattern of parallel evolution in sticklebacks is reduction of skeletal armor, which has occurred in numerous freshwater locations around the world. New genetic and genomic tools for the threespine stickleback have made it possible to identify genes that underlie loss of different elements of the skeletal armor. Previous work has shown that regulatory mutations at the *Pitx1* locus are likely responsible for loss of the pelvic structures in independent stickleback populations from North America and Iceland. Here we show that the *Pitx1* locus is also likely to underlie pelvic reduction in a Scottish population of threespine stickleback, which has apparently evolved pelvic reduction under a different selection regime than the North American populations.

Although the selective forces that contribute to adaptive phenotypic variation are beginning to be understood, very little is known about the genetic changes on which selection acts during phenotypic evolution (Orr 2005). The threespine stickleback (*Gasterosteus aculeatus*) is an excellent model system in which to integrate ecological and genetic studies to gain a greater understanding of the process of phenotypic evolution (Foster and Baker 2004; Kingsley et al. 2004; Gibson 2005; Kingsley and Peichel 2007). This small teleost fish displays such great phenotypic variation that different forms were originally classified as more than 40 different species, with some of the most obvious phenotypic changes in stickleback populations occurring in skeletal armor. In most locations, these fish are encased in bony armor that consists of lateral plates, dorsal spines, and 2 pelvic spines supported by a pelvic girdle. However, armor reduction has occurred in freshwater locations that have only existed since the retreat of the glaciers less than 20 000 years ago. In particular, pelvic reduction has occurred in a small number of independent locations ranging from the northwest coast of Scotland to Iceland to several sites along the Pacific coast of North America (Bell 1974, 1987; Moodie and Reimchen

1976; Campbell 1979; Bell et al. 1993; Shapiro et al. 2004). The evolution of armor reduction is associated with specific predation regimes and low calcium levels in North American populations (Reimchen 1980; Bell et al. 1993) but is solely associated with low calcium levels in Scottish populations (Giles 1983).

The development of genetic and genomic tools for threespine stickleback has made it possible to identify the genetic and molecular basis of phenotypic variation in natural populations of this species (Peichel 2005; Kingsley and Peichel 2007). Genome-wide linkage mapping carried out using Canadian pelvic-reduced populations identified the *Pitx1* gene as a candidate locus of large effect for pelvic size (Shapiro et al. 2004). Genetic crosses suggest that the same locus also underlies pelvic reduction in Icelandic and Alaskan threespine stickleback populations (Cresko et al. 2004; Shapiro et al. 2004) as well as pelvic reduction in a closely related species, the ninespine stickleback (Shapiro et al. 2006). In Canadian and Scottish pelvic-reduced populations, gene expression studies have demonstrated that *Pitx1* is not expressed in the developing pelvic region (Cole et al. 2003; Shapiro et al. 2004). However, mapping

Table 1. Pelvic spine number in F₂ cross fish

	Pelvic spine number		
	0	1 ^a	2
F ₂ family	0	1 ^a	2
Family 1 F ₂ S (N = 66)	17	1	48
Family 2 F ₂ S (N = 36)	12	1	23
Family 3 F ₂ S (N = 38)	9	1	28
Family 4 F ₂ S (N = 37)	12	2	23

The number of F₂ fish in a family with 0, 1, or 2 pelvic spines is indicated.

^a One of the F₂S from Family 4 was missing its left pelvic spine; all additional fish with 1 pelvic spine were missing the right pelvic spine.

studies had not been carried out in the Scottish population, so it was unknown whether the loss of *Pitx1* expression in this population is genetically linked to the *Pitx1* locus or results from a mutation in an unlinked gene that regulates *Pitx1* expression. Here we show that the pelvic reduction phenotype is tightly linked to the *Pitx1* locus in a Scottish population. Although our data do not rule out the possibility that the loss of *Pitx1* expression in this Scottish population results from changes in a closely linked gene that regulates *Pitx1* expression, this result provides evidence that changes at or near the *Pitx1* locus have occurred in multiple, independent stickleback populations with pelvic reduction.

Materials and Methods

Fish

Sticklebacks from the River Kelvin (Glasgow, Scotland) and Loch Fada (North Uist, Scotland) were caught in February 2003 and transported in aerated 25-gallon buckets to the Glasgow University Field Station (SCENE), Loch Lomond-side. Fish from a single population were kept together in 1.3 × 1.3 m, 500-l flow-through indoor tanks at a maximum density of 40 fish per tank. Several large plastic plants were placed in each tank as a refuge for fish to hide in. Fish were fed frozen and live bloodworm (*Chironomus* spp.) and frozen water fleas (*Daphnia* spp.) ad libitum and maintained on an ambient photoperiod at ambient loch water temperature (6 ± 2 °C).

Crosses

For genetic mapping, 1 Loch Fada female that had no pelvic spines or pelvic girdle structures (pelvic score = 0; Bell et al. 1993) was crossed with 1 River Kelvin male that had 2 pelvic spines and complete pelvic girdle structures (pelvic score = 8) to produce 40 F₁ offspring. Eight of these F₁ fish were intercrossed, resulting in 177 F₂ progeny from 4 F₂ families (Table 1). The F₁ parents of all families had complete pelvic girdles and 2 pelvic spines (pelvic score = 8), except for the Family 4 F₁ father, which had a complete pelvic girdle but was missing the right pelvic spine (pelvic score = 7).

Males in breeding condition were moved to sandy bottomed breeding tanks (45 × 27 × 15 cm) with nesting material. After nest construction, a gravid female was placed in the tank and the pair was left to breed naturally. After spawning, the female was removed and the fertilized eggs placed in an incubator and artificially oxygenated at 16 °C. Males rebuilt a nest within 1–3 days, and a gravid female was returned to the tank to breed again. Fry were fed Liquifry No1 (INTERPET) for 1 week after hatching and then maintained on a mixture of enriched AF high-grade *Artemia* (INVE AQUACULTURE nv) and chopped bloodworm. At 8 weeks, fry were moved to small holding tanks (25 × 20 × 45 cm) and kept in family groups of 10–15 fish.

Phenotypic Analysis

Parental and F₁ adults and F₂ fish at 24 weeks after hatching were killed with an overdose of anesthetic (benzocaine). Fins were clipped for DNA extraction, and the bodies were preserved in 100% ethanol. The number of pelvic spines was counted, and measurements of standard length (tip of snout to end of caudal peduncle), pelvic spine lengths (tip to anterior edge of spine), and ventral pelvic girdle length (between spines to posterior tip of girdle) were made with calipers to the nearest 0.1 mm. Asymmetry was calculated as the ratio of the length of the left pelvic spine to the combined length of the left and right pelvic spines, such that a value of 0.5 indicates perfect symmetry, a value of 0.0 indicates the loss of the left pelvic spine, and a value of 1.0 indicates loss of the right pelvic spine.

Genetic Analysis

Six microsatellite markers (*Stn76*, *Stn257*, *Stn80*, *Stn82*, *Stn336*, *Stn342*) previously mapped to linkage group 7 (LG7) were used for genotyping (Shapiro et al. 2004). These markers are found in the *G. aculeatus* sequence assembly BROAD S1 (http://www.ensembl.org/Gasterosteus_aculeatus/index.html) at approximately 5.22 Mb (*Stn76*), 25.10 Mb (*Stn257*), 26.40 Mb (*Stn80*), and 26.66 Mb (*Stn82*) on LG7, and on scaffold 76 (*Stn336*, *Stn342*), which has not been assigned to a LG in the sequence assembly. These markers were used to genotype 177 F₂ progeny as well as the grandparents and F₁ parents, using previously described polymerase chain reaction (PCR) conditions (Peichel et al. 2001). However, the DNAs of 2 fish were not included in the genetic analysis (Loch Fada grandmother and Family 2 F₁ mother). PCRs were analyzed on an ABI 3100 (Applied Biosystems, Foster City, CA). Two people independently determined genotypes by manually calling the allele sizes and segregation patterns, which were visualized with ABI GeneMapper 3.7 (Applied Biosystems). A map of LG7 was generated in JoinMap 3.0 (Van Ooijen and Voorrips 2001) using the default settings, and interval mapping of 5 pelvic traits was performed in MapQTL 4.0 (Van Ooijen et al. 2002) using default settings. Likelihood of odds (LOD) significance thresholds were determined for each trait by permutation tests in MapQTL 4.0 using a chromosome-wide

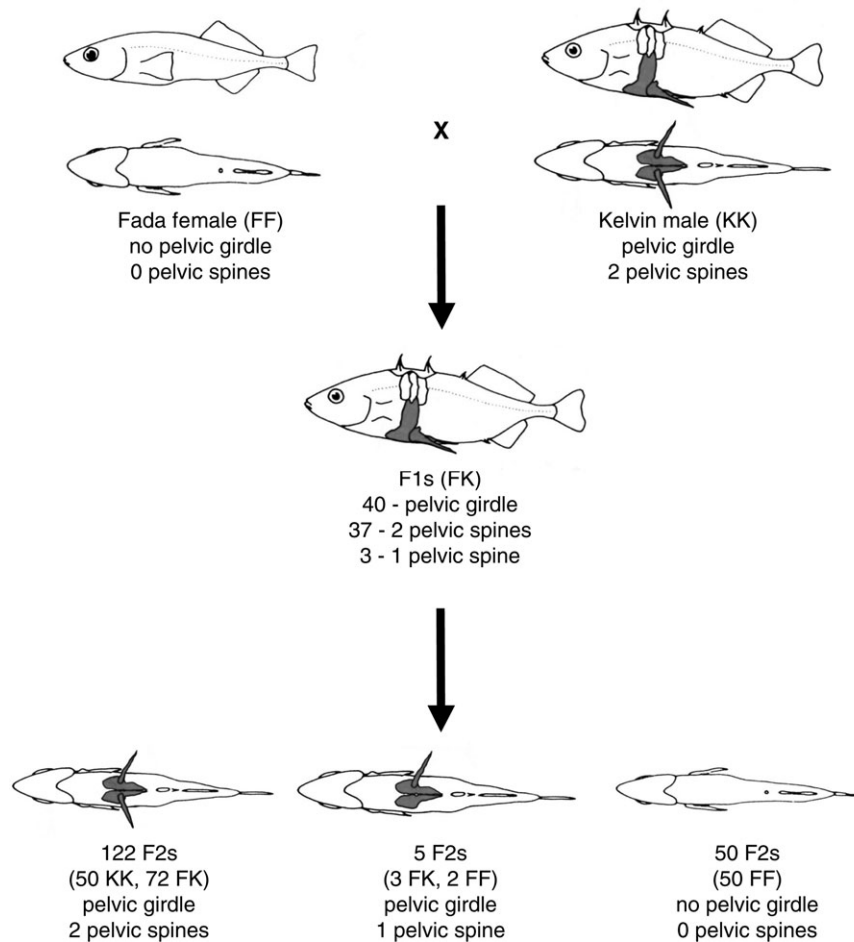


Figure 1. Intercross for genetic mapping of pelvic reduction. Line drawings of representative fish, with pelvic structures highlighted in red are shown for the parental, F₁, and F₂ generation. Both lateral and dorsal views of the parental fish are shown to highlight the complete absence of pelvic structures (girdle and spines) in the Loch Fada mother. The ascending branch of the pelvic girdle can be seen in the lateral view of the River Kelvin father, and the ventral pelvic girdle and 2 pelvic spines can be seen in the ventral view of the River Kelvin male. Only ventral views of representative F₂ fish are shown to emphasize the different pelvic spine classes. Genotypes at the *Pitx1* microsatellites (*Stm 336/Stm 342*) are indicated in parenthesis.

significance threshold of $\alpha = 0.01$ for $n = 1000$ permutations. Because only 1 chromosome was genotyped and tested, the chromosome-wide and genome-wide LOD significance thresholds are equivalent.

Results and Discussion

We crossed a female completely lacking pelvic spines and pelvic girdle structures from an armor-reduced Scottish population (Loch Fada) known to lack pelvic *Pitx1* expression (Cole et al. 2003) to a male with a complete pelvic complex from a robustly armored Scottish population (River Kelvin). From this cross we generated 40 F₁ progeny, which all had complete pelvic structures, except for 3 fish that had complete pelvic girdles, but showed loss of the right pelvic spine. Four pairs of full-sib F₁ hybrid fish were

intercrossed to generate 4 F₂ families, producing a total of 177 F₂ progeny (Figure 1 and Table 1). In the F₂ progeny, there was an approximate 3:1 ratio of progeny with complete pelvic girdles and either 2 or 1 pelvic spines to progeny with no pelvic structures (Figure 1; $\chi^2 = 0.996$, $P = 0.318$, degrees of freedom = 1). These data suggested that there might be a major locus responsible for loss of the pelvic complex in this Scottish population, as seen in Canadian and Alaskan populations (Cresko et al. 2004; Shapiro et al. 2004).

To determine whether *Pitx1* is genetically linked to the major pelvic reduction locus in the Loch Fada population, we genotyped F₂ fish with 6 informative microsatellite markers from stickleback LG7, including 2 markers in the *Pitx1* gene, to which the major pelvic locus has been mapped in other populations (Cresko et al. 2004; Shapiro et al. 2004). Of the 50 F₂ fish that had complete loss of

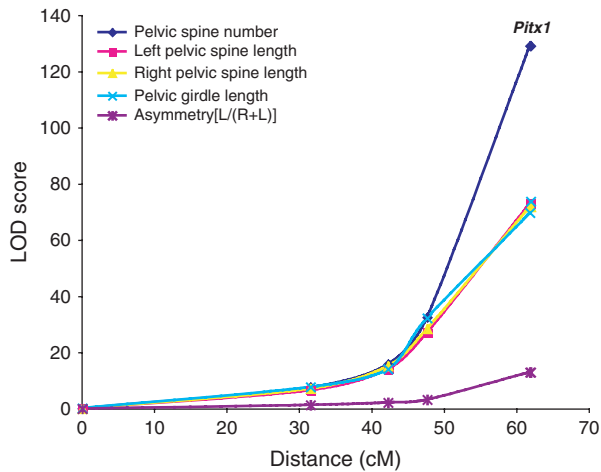


Figure 2. Interval mapping of pelvic traits on LG7. LOD scores are plotted as a function of the position in cM of markers: *Stn76* (0.0 cM), *Stn257* (31.5 cM), *Stn80* (42.2 cM), *Stn82* (47.6 cM), *Stn336/Pitx1* (61.8 cM), and *Stn342/Pitx1* (61.8 cM), which are indicated by points along the curve.

pelvic structures, all inherited 2 Fada alleles at the *Pitx1* markers (Figure 1). Interval mapping showed that the *Pitx1* locus explains between 85.1% and 96.6% of the variance in pelvic spine length and number, as well as pelvic girdle length, with LOD scores between 72.0 and 129.3 (Figure 2 and Table 2). These mapping data, together with the previous evidence for lack of pelvic *Pitx1* expression in the Fada population (Cole et al. 2003), provide strong evidence that mutations at the *Pitx1* locus or at a tightly linked gene that regulates *Pitx1* expression, rather than an unlinked gene that regulates *Pitx1* expression, are largely responsible for pelvic reduction in this Scottish population.

The high percentage of phenotypic variance explained by the *Pitx1* locus in these crosses suggests that modifier loci with detectable effects on pelvic phenotype are not segregating in the Loch Fada population. Furthermore, the

Table 2. Effect of *Pitx1* on pelvic phenotypes

Trait	LOD	PVE	Phenotype means		
			KK	FK	FF
Pelvic spine number	129.3	96.6	2.00	1.96	0.04
Left pelvic spine length	73.4	86.0	4.29	3.69	0.08
Right pelvic spine length	72.0	85.1	4.29	3.58	0.00
Pelvic girdle length	73.4	87.0	5.50	5.13	0.10
Asymmetry	13.2	38.0	0.50	0.51	1.00

The LOD score and percent variance explained (PVE) at the *Pitx1* locus (*Stn 336/Stn 342*) are shown for each of 5 pelvic traits measured. For each trait, a LOD score of greater than 3.0 is considered significant by permutation testing using a chromosome-wide significance threshold of $\alpha = 0.01$. Mean phenotypic values of each trait were calculated for progeny that inherited 2 *Pitx1* alleles from the Kelvin grandparent (KK), 2 *Pitx1* alleles from the Fada grandparent (FF), or 1 *Pitx1* allele from each (FK).

Pitx1 locus explains similar levels of phenotypic variance across all 4 F₂ families, suggesting that we are not detecting within-population genetic variation for pelvic reduction (Supplementary Table 1). These results differ from mapping studies in a Canadian pelvic-reduced population, where the *Pitx1* locus explained 46.8% and 65.3% of the variance in pelvic girdle length and pelvic spine length, respectively, and 4 modifier loci of smaller effect were genetically mapped (Shapiro et al. 2004). Modifier loci with minor effects on pelvic phenotypes are also segregating in Alaskan populations (Cresko et al. 2004). Consistent with our findings for pelvic reduction in the Loch Fada population, modifier loci for armor plate reduction were identified in a Canadian but not in a Californian threespine stickleback population (Colosimo et al. 2004), suggesting that the presence of modifiers of smaller effect may vary between stickleback populations.

Interestingly, 3 of the F₁ fish and 4 of the F₂ fish had complete pelvic structures, except for loss of the right pelvic spine, whereas a single F₂ fish had complete pelvic structures except for loss of the left pelvic spine (Figure 1 and Table 1). This left-biased asymmetry is consistent with previous results in both natural populations (Bell 1974, 1987; Moodie and Reimchen 1976; Bell et al. 1985, 2007) and laboratory crosses (Cresko et al. 2004; Shapiro et al. 2004). This observation is also consistent with the fact that the loss of *Pitx1* can be partially compensated by *Pitx2*, which is preferentially expressed on the left side of the body (Marcil et al. 2003). Of the 5 F₂ fish with a single pelvic spine, 2 were homozygous for the Fada alleles at the *Pitx1* markers, whereas 3 (including the fish that had lost the left pelvic spine) were heterozygous at the *Pitx1* markers (Figure 1). Interval mapping revealed that the *Pitx1* locus explains 38% of the asymmetry in pelvic spine length, with a significant LOD score of 13.2 (Figure 2 and Table 2), consistent with previous results (Shapiro et al. 2004).

We thus conclude that pelvic reduction in the Scottish Loch Fada population, which has likely evolved as a result of selection under low calcium levels rather than lack of piscivorous fish predators (Giles 1983), represents a case of parallel evolution at or near the *Pitx1* locus. Recent genetic studies in a number of taxa have uncovered other examples of parallel evolution involving the same locus. For example, the *yellow* gene controls changes in wing pigmentation in different *Drosophila* species (Gompel and Carroll 2003; Gompel et al. 2005; Prud'homme et al. 2006), the *shavenbaby/ovo* gene is responsible for changes in hair patterns in *Drosophila* species (Sucena et al. 2003), coding mutations in the *Mel1r* gene result in melanism in many vertebrate lineages (Theron et al. 2001; Eizirik et al. 2003; Nachman et al. 2003; Mundy et al. 2004; Rosenblum et al. 2004; Hoekstra et al. 2006; Römpler et al. 2006), and the *Oca2* gene is responsible for albinism in fish and mammals (Protas et al. 2006). The recurrent use of a small set of genes during morphological evolution suggests that genetic and/or developmental bias may play an important role in adaptation. Additional studies to identify the molecular changes that give rise to loss of pelvic structures in

Canadian, Alaskan, Icelandic, and Scottish populations will allow us to determine whether parallel evolution of pelvic reduction results from standing genetic variation in the ancestral marine population as found for loss of bony lateral plates (Colosimo et al. 2005) or from independent mutations, implicating genetic and/or developmental bias as a key factor in the evolution of pelvic reduction in stickleback.

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Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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