Current Biology 19, 1140–1145, July 14, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.05.029

Report

The Genetic Architecture of Skeletal Convergence and Sex Determination in Ninespine Sticklebacks

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Summary

The history of life offers plentiful examples of convergent evolution, the independent derivation of similar phenotypes in distinct lineages [1]. The emergence of convergent phenotypes among closely related lineages (frequently termed "parallel" evolution) is often assumed to result from changes in similar genes or developmental pathways [2], but the genetic origins of convergence remains poorly understood. Ninespine (*Pungitius pungitius*) and threespine (*Gasterosteus aculeatus*) stickleback fish provide many examples of convergent evolution of adaptive phenotypes, both within and between genera. The genetic architecture of several important traits is now known for threespine sticklebacks [3–10]; thus, ninespine sticklebacks provide a unique opportunity to critically test whether similar or different chromosome regions control similar phenotypes in these lineages. We have generated the first genome-wide linkage map for ninespine sticklebacks and used quantitative trait locus mapping to identify chromosome regions controlling several skeletal traits and sex determination. In ninespine sticklebacks, these traits mapped to chromosome regions not previously known to control the corresponding traits in threespine sticklebacks. Therefore, convergent morphological evolution in these related, but independent, vertebrate lineages might have different genetic origins. Comparative genetics in sticklebacks provides an exciting opportunity to study the mechanisms controlling similar phenotypic changes in different animal groups.

Results and Discussion

Genome-wide Linkage Map

The last several years have witnessed substantial progress in characterizing the genetic basis of adaptive diversity in natural populations and species. In threespine sticklebacks, the development of new genetic and molecular tools has made it possible to identify major loci controlling repeated evolution of changes in skin color, the pelvis, the operculum, and the number and size of armor plates in populations that colonized new lakes and streams generated by widespread deglaciation beginning about 20,000 years ago [3–6, 9, 10]. An emerging theme of genetics studies in threespine sticklebacks is that the same genes or chromosome regions underlie similar phenotypes in multiple natural populations; examples include the major effects of *Pitx1* [4, 6, 11, 12] and *Ectodysplasin (Eda)* [5, 6, 8] in the evolution of derived pelvic and armor phenotypes, respectively, throughout the range of this species. Development of comparable genetic resources for ninespine sticklebacks makes it possible for us to critically compare the genetic basis of convergent evolution in a fish group that has also evolved a number of similar interesting morphological and physiological changes (Figure 1) but that last shared a common ancestor with threespine sticklebacks well over 13 million years ago [13].

To generate a genome-wide linkage map for quantitative trait locus (QTL) studies, we produced a *Pungitius pungitius* genomic library, screened it with a probe for microsatellite repeats, sequenced individual clones, and designed PCR primers that could amplify individual microsatellite repeat regions from ninespine stickleback genomic DNA samples. We typed 212 microsatellite markers (169 derived from ninespine sticklebacks and 43 from threespine sticklebacks) on 120 F1 progeny from a cross between Canadian and Alaskan ninespine sticklebacks, both lacking pelvic structures (Figures 1C and 1E). The female parent came from Fox Holes Lakes (Northwest Territories, Canada), which is monomorphic for total absence of the pelvis [14]. The male parent came from an unnamed creek on Pt. MacKenzie (Matanuska-Susitna Borough, south-central Alaska), where ninespine sticklebacks are polymorphic for pelvic phenotypes (average pelvic score for the Pt. MacKenzie population is 1.96 on a scale [15] that ranges from 0 [bilateral absence of pelvic structures] to 8 [all four pelvic elements are present on both sides]). The combined ninespine and threespine markers defined 30 genetic linkage groups (LGs), comprising 190 markers (151 from ninespine sticklebacks, 39 from threespine sticklebacks) and spanning a total genetic distance of 957.8 cM (Figure S1). Because cytological studies show that *Pungitius* has 21 chromosomes [16–18], we expect some current linkage groups to coalesce with others as additional markers are added to the map.

To compare LGs in ninepsine and threespine sticklebacks, we examined map locations of the 39 markers that could be amplified from genomic DNA in both species. We also used BLAST searches to compare the unique sequences from the 151 newly isolated and mapped ninespine stickleback markers with an initial genome assembly for the threespine stickleback (http://www.ensembl.org/Gasterosteus_aculeatus/index.html). In all, 88.7% (134/151) of ninespine stickleback marker sequences mapped to unique threespine stickleback chromosome scaffolds, 2.0% (3/151) mapped to unassembled scaffolds, and the remaining 9.3% (14/151) either produced no significant BLAST results or mapped to multiple genomic scaffolds (Table S1). At least 50% of markers in each ninespine linkage group were associated with a single threespine chromosome (87.9% of markers overall, mean of 85.2% of markers per linkage group). These results suggest that synteny has been well conserved between the two genera, both of which

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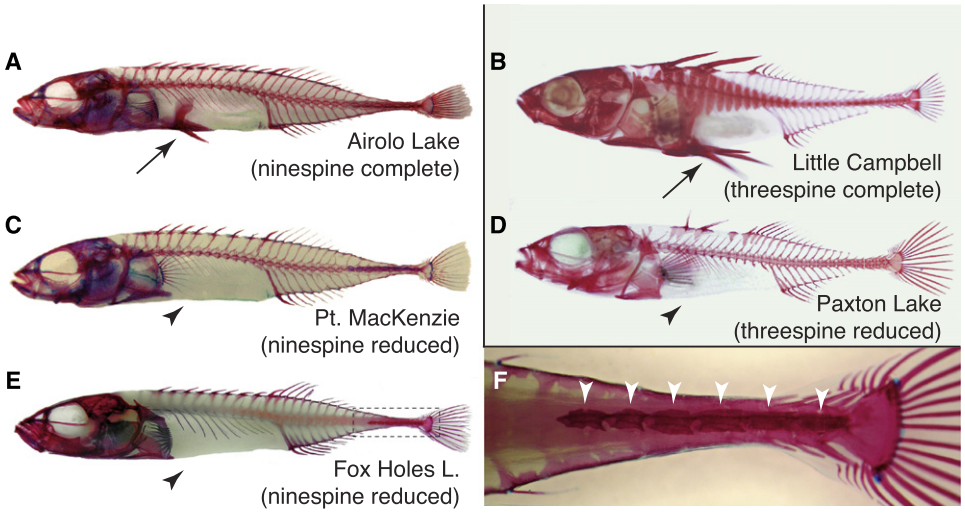

Figure 1. Convergent Skeletal Evolution in Ninespine and Threespine Sticklebacks

Reduction and loss of the pelvic (hind) fin has evolved in multiple populations of both ninespine and threespine sticklebacks.

(A and B) Ninespine (A) and threespine (B) sticklebacks with complete pelvic skeletal structures (arrow) from Airolo Lake, Alaska, and Little Campbell River, British Columbia.

(C and E) Ninespine sticklebacks missing all pelvic structures (arrowhead) from Point MacKenzie, Alaska, and Fox Holes Lakes, Northwest Territories. These two populations were used in the mapping cross.

(D) A similar pelvisless phenotype occurs in the benthic threespine sticklebacks of Paxton Lake, British Columbia.

(F) Enlargement of boxed area in (E) showing details of the caudal portion of the bony armor (arrowheads), which varies in numbers of plates among fish from different populations and in our laboratory cross. All specimens were cleared by digestion in trypsin and stained in alizarin red S for visualization of ossified skeletal structures. Photographs are not to scale.

have 21 cytologically visible chromosomes [16]. For ease of comparing results between species, we numbered linkage groups in the ninespine genetic map to match the syntenic linkage group in the threespine map.

Comparative Mapping of Pelvic Reduction

A dramatic example of convergent evolution between populations and genera of sticklebacks is the reduction or loss of the pelvic (hind fin) skeleton (Figure 1). The pelvis is present in all marine and most freshwater populations of threespine and ninespine sticklebacks, but it has been lost repeatedly in several freshwater populations, probably as an adaptation to local predators and water chemistry [4, 14, 15, 19–25]. Previous studies of threespine sticklebacks have identified one QTL of major effect on LG7; this QTL controls more than 50% of the variation in pelvic size in crosses from diverse geographic locations, including British Columbia, Alaska, Iceland, and Scotland [4, 6, 12]. Mapping, sequencing, and expression studies suggest that this major QTL corresponds to the *Pitx1* locus [4, 12, 26], a homeodomain transcription factor that is expressed in developing hindlimbs but not forelimbs of vertebrates [27–29]. Previous complementation and in situ studies show that Fox Holes Lakes ninespine sticklebacks have recessive genetic changes that also reduce *Pitx1* expression in the pelvis [30].

Presence or absence of a pelvic skeleton segregates in a 1:1 Mendelian ratio in our ninespine stickleback cross (Figure 2). Of the 120 progeny analyzed, 59 had complete pelvic skeletons (bilateral presence of an anterior process, posterior process, ascending branch, and spine [31]), eight had partial skeletons (six of these had fewer than half of the normal structures), and 53 lacked all pelvic structures. The binary, qualitative trait of presence versus absence of the pelvic complex (partial phenotypes excluded) mapped to LG4 with a peak LOD score of 82.16 (Figure 2D; Tables S2 and S4). Detailed analysis of marker genotypes shows that the striking dimorphism in this cross originates from the Alaskan male parent—inheritance of one Alaskan parental LG4 haplotype is usually associated with a complete pelvis in the F1 progeny, whereas inheritance of the other Alaskan haplotype is usually associated with the absence of pelvic structures (Table S4). Thus, the Alaskan male parent of the cross, which comes from a population that is polymorphic for pelvic phenotypes, was heterozygous for a dominant allele for pelvic reduction. The phenotypic effect of the LG4 region in the current cross is as large as that reported previously for the *Pitx1* (LG7) region in threespine sticklebacks [4, 6, 12], but this region maps to a completely different linkage group. This QTL on LG4 is unlinked to a marker in an intron of the ninespine stickleback *Pitx1* gene (Pun319) and to two markers on threespine stickleback BAC clones containing the *Pitx1* gene (Stn430, Stn431; Figure 2E and Figure S1). The threespine stickleback genomic region that corresponds to the pelvic-reduction region in the ninespine cross contains several genes with known roles in limb and fin development; such genes include members of the *Fgf*, *Msx*, and *Wnt* families. We are currently investigating the potential roles of these candidate genes in pelvic reduction in the Pt. MacKenzie population.

Ninespine sticklebacks from the Pt. MacKenzie population show a key morphological difference in comparison to most other reduced-pelvis populations. Most extant and fossil threespine stickleback populations [32], mice with knockouts in the *Pitx1* gene [33], and Florida manatees with vestigial pelvic structures [30] show greater pelvic reduction on the right than the left side. In contrast, the Pt. MacKenzie ninespine sticklebacks tend to show greater pelvic reduction on the left than the right side. Our linkage studies provide the first genetic evidence that populations with different types of

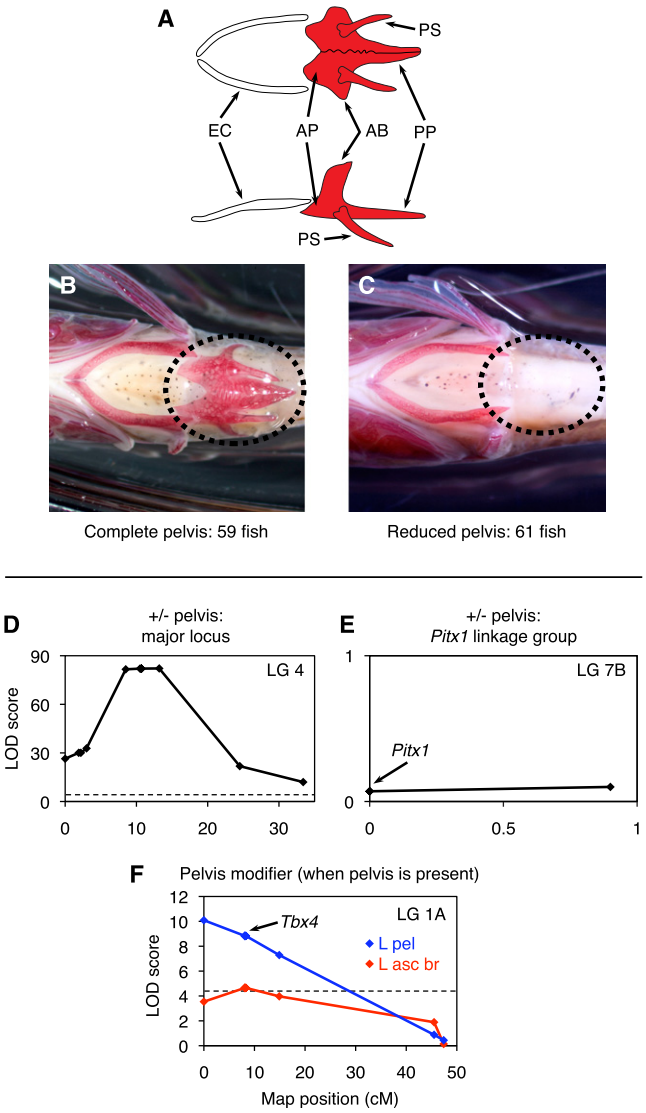

Figure 2. Pelvic-Reduction Maps to LG4, Not to *Pitx1*, in a Ninespine Stickleback Cross

(A) Morphology of the ninespine stickleback pelvis and ectocoracoid in ventral (top) and lateral (bottom) views. A complete pelvis shows bilateral presence of the anterior process (AP), posterior process (PP), ascending branch (AB), and pelvic spine (PS). Anterior to the pelvis is the ectocoracoid bone (EC) of the pectoral girdle.

(B and C) The 120 progeny showed a 1:1 ratio of (B) complete to (C) reduced pelvic phenotypes. Anterior is to the left in both images.

(D) A QTL on LG4 controlled the presence versus absence of the pelvis. Only informative markers (polymorphic in the Alaskan male parent) are shown. The plateau of the LOD peak is due to low recombination between LG4 haplotypes in the Alaskan parent of the cross.

(E) The linkage group containing *Pitx1* did not have a significant effect on pelvic phenotype.

(F) Restricted multiple QTL mapping analysis detected an additional QTL interval influencing the height of the left ascending branch (L asc br; red) and length of the pelvic girdle (L pel; blue), and this interval includes the *Tbx4* gene, a transcription factor involved in hindlimb development [28]. The length of the pelvic girdle was measured from the anterior tip of AP to the posterior tip of PP. Dashed lines indicate the LOD significance threshold (95% genome-wide level of $\geq$4.5 in D [59] and $\geq$ 4.3 in F; not shown in E for the purpose of limiting the LOD scale and preserving the visibility of the plot). Diagrams in (A) were modified from [31].

Table 1. Comparison of QTL for Skeletal Traits and Sex Determination in Ninespine and Threespine Sticklebacks

Trait	Ninespine LG	Threespine LG	References
Pelvis (complete versus reduced)	4	7	[4, 6, 12]
Ascending-branch height	1, 4	7, 10	[4]
Pelvic-girdle length	1*, 4*	1*, 2, 4*, 7	[4]
Pelvic-spine length	4*	2, 4*, 7, 8	[3, 4]
Lateral-plate number	12	4, 7, 10, 26[a]	[5, 6, 8]
Sex determination	12	19	[7]

Similar mapping results are marked with an asterisk.
[a] Chromosome 21 in threespine stickleback genome assembly.

directional asymmetry have changes in different major genes controlling pelvic reduction. Approximately 10% of threespine stickleback populations with extensive pelvic reduction show greater reduction on the left than the right side [32]. It will be interesting to see whether pelvic reduction in these populations maps to the same region detected in Pt. MacKenzie ninespine sticklebacks.

Although the major QTL for pelvic reduction in our ninespine cross is clearly distinct from the *Pitx1* locus, the position of the QTL on LG4 is in a region similar to a pelvic modifier QTL that controls less than 6% of the variation in the length of the pelvic spine girdle in a cross between marine (complete pelvis) and reduced-pelvis threespine sticklebacks [4] (Table 1). It is possible that similar genes contribute to pelvic reduction in both threespine and ninespine sticklebacks, but the magnitude of their phenotypic effects differs dramatically between genera. The large impact of the LG4 region in the ninespine fish probably depends in part on a sensitized genetic background in the cross between Pt. MacKenzie and Fox Holes Lakes sticklebacks, where all F1 progeny also inherited pelvic reduction alleles from the Fox Holes Lakes parent [30]. The LG4 region in the current cross has a larger phenotypic effect than *Eda*, *Pitx1*, or *Kit ligand* (*Kitlg*) genes in threespine sticklebacks; each of these three genes has been successfully isolated by mapping or positional cloning studies [4, 8, 10]. Ninespine sticklebacks should thus provide a very useful system for identifying additional loci controlling major evolutionary phenotypes in natural populations.

A single region on ninespine LG4 largely controls the presence-versus-absence pelvic phenotype, yet other chromosomal regions control quantitative variation in pelvic size in those progeny that do have a pelvis. For example, we identified a region on LG1A that controls up to 33.2% of the variation in left and right pelvic structures; there was a more pronounced effect on the left than the right side (Figure 2; Figure S1 and Tables S2 and S4). The LG1A QTL in the ninespine cross overlaps the broad location of a QTL interval that controls approximately 6% of the variance in pelvic-girdle length in a threespine stickleback cross [4].

Notably, variation at the major and modifier pelvic loci reveals cryptic genetic variation (CGV) in the wild Pt. MacKenzie population. CGV is thought to be an important and pervasive, yet underappreciated, factor in the response of organisms to mutation, selection, and disease [34]. Both parents of our cross had similar pelvisless phenotypes, yet half of their progeny developed complete pelvises on the hybrid genetic background. Most of the fish in the wild Pt. MacKenzie population also exhibit extreme pelvic reduction, so much of the variation in the major and modifier pelvic loci may remain hidden except under extreme environmental conditions, or in response to

genetic perturbations such as hybridization with other genetic backgrounds, as is the case on our cross (reviewed in [34]). This study provides a dramatic example of the phenotypic diversity that can result when admixture occurs between different outbred genetic backgrounds.

A Novel Chromosomal Region Controls Lateral Armor in Ninespine Sticklebacks

Other skeletal traits mapped to different regions of the genome in ninespine sticklebacks relative to threespine sticklebacks (Table 1; Figure S1 and Tables S2–S5). In threespine sticklebacks, variation in lateral plates maps to the *Eda* locus on LG4 [5, 6, 8]. We mapped two markers in and around the *Eda* locus in our ninespine cross; these included Stn364 (located in an intron of the *Eda* gene itself) and the closely linked Stn361 marker (located 16 kb away, just 5′ of the *Eda* locus). Both markers mapped to LG4, in a chromosome region that did not have significant effects on plate phenotypes, in the ninespine stickleback cross. Instead, variation in lateral-plate number in the ninespine stickleback cross (Figure 1F) mapped to LG12, the same chromosome region that determines sex (see below). This linkage group accounted for nearly one-third (30.1% left side and 28.4% right side) of the variance in plate number. Notably, unlike the major pelvic locus on LG4, segregation of different alleles on LG12 from both the Alaskan and Northwest Territories parents had significant effects on plate phenotypes (Tables S3 and S5). The armor QTL on LG12 is also distinct from all known chromosome regions that have smaller quantitative effects on armor phenotypes in threespine sticklebacks with reduced numbers of plates (Table 1).

The Sex-Determination Locus Differs between Stickleback Genera

Several different mechanisms underlie sex determination among teleost fishes, and under normal conditions sex can be determined by genetic and/or environmental cues [35, 36]. Sex determination in threespine sticklebacks behaves as a simple Mendelian trait that maps to LG19 [7]. Sex determination in ninespine sticklebacks also behaves as a Mendelian trait, but it maps to LG12, in a completely different region of the genome relative to markers closely linked to the sex-determining region in threespine sticklebacks (Stn186, Stn194) [7] (Table 1; Figure S1 and Tables S3 and S5).

The sex-determining region of LG19 in threespine sticklebacks shows striking differences in recombination rates in male versus female meiosis [7]. Similarly, LG12 in the ninespine stickleback cross covers approximately 13 cM largely because of a lack of recombination in male meioses (Figures S1 and S2). In contrast, when female meioses were analyzed independently of male meioses, the genetic distances between markers were greater, and LG12 covered 27 cM (Figure S2B). Thus, although different chromosomes are involved in sex determination in the two genera, the linkage group bearing the sex-determining region in ninespine sticklebacks has some of the same recombination characteristics as the threespine stickleback Y chromosome.

The genomic positions of the major sex-determining loci are different in threespine and ninespine sticklebacks, yet it is possible that the same molecular mechanisms determine this fundamental trait in both genera. For example, both genera may have inherited the same sex-determination mechanism from a common ancestor, but the gene(s) underlying this mechanism may be located on different chromosomes because of different evolutionary translocations, as has occurred in salmonids [37]. Identification of the genes controlling sex determination on LG12 of ninespine sticklebacks and chromosome 19 of threespine sticklebacks will permit a direct test of this hypothesis.

Multiple Phenotypic Traits Cluster on the Sex Chromosome

Several other phenotypic traits, including jaw length, head length, orbit (eye) diameter, and pectoral fin length, mapped to LG12 in ninespine sticklebacks (Figure S1 and Tables S3 and S5). With the exception of pectoral-fin length, the phenotypic means for each of these traits were larger in male than in female fish. Sexual dimorphism in head size and other skeletal traits has previously been demonstrated for wild populations of *Pungitius* [38, 39] and for wild and lab-bred *Gasterosteus* [40–45]. Clustering of phenotypic traits on the sex chromosome could be due either to pleiotropic effects of a single sex-determining locus or to multiple loci controlling sexually dimorphic traits that are physically and genetically linked on the sex chromosomes. When we repeated our analysis of sex-linked traits by using residuals with the average effect of sex removed, we no longer detected significant QTL on LG12, suggesting that most LG12 QTL are detecting male-female differences rather than effects of alternative chromosomes within males or females. However, we did detect significant differences between the two female haplotypes on LG12 for lateral-plate phenotypes by using the transformed data ($p < 0.05$, ANOVA with Tukey's Multiple Comparison Test), consistent with our QTL mapping of this trait described above.

Linkage between the primary sex-determining locus and genes with differential effects in males and females is thought to be a key feature that drives sex-chromosome evolution, including the accumulation of inversions and sequence divergence that suppresses recombination between the sex-determining locus and neighboring genes [46]. Furthermore, the linkage between sex determination and LG12 in ninespine sticklebacks and at least one other stickleback species (*Gasterosteus wheatlandi*, the black-spotted stickleback) suggests that this chromosome "might have an abundance of genes with differential fitness effects in males and females and thus be predisposed to becoming a sex chromosome" [18]. The distinct linkage groups that control sex determination in threespine and ninespine sticklebacks will provide an excellent system for comparing mechanisms of both sex determination and sex-chromosome evolution in closely related lineages.

Genetics of Convergent Evolution

Several genetics studies have demonstrated that the same genes probably underlie similar changes among different animal lineages. For example, in *Drosophila*, *Ultrabithorax* and *Ovo/shavenbaby* control similar changes among different species in leg and abdominal trichome patterns, respectively, and repeated changes at the *yellow* locus control similar wing pigmentation in different species [47–50]. Among vertebrates, evolution of similar pigmentation phenotypes resulting from changes in the *Melanocortin 1 receptor* (*Mc1r*) in mammals, birds, and reptiles (reviewed in [51]), in *Oculocutaneous albinism 2* (*Oca2*) in multiple populations of cavefish [52], and in *Kitlg* in both sticklebacks and humans [10] demonstrate that independent changes in the same gene can generate broadly similar phenotypes in multiple lineages. In contrast, other examples of convergent morphological evolution appear to depend on different genetic mechanisms. For instance, complementation crosses suggest that regressive eye loss in

blind Mexican cavefish has occurred by different mechanisms in different cave populations [53, 54]. Although variant alleles of *Mc1r* control pigmentation phenotypes in the beach mouse (*Peromyscus polionotus)* and rock pocket mouse (*Chaetodipus intermedius*), exceptions to this genetic trend are known for each species [55–57]. Likewise, different genes in different species of *Drosophila* control similar changes in abdominal pigmentation [58].

Because recent genetic studies in threespine sticklebacks show that similar chromosomal regions control similar phenotypes in many different populations [4–6, 8, 10, 30], we recognized at the inception of this study that genetic mapping in ninespine sticklebacks might largely identify the same chromosomal regions. However, for every trait we examined, we found that the major loci controlling skeletal traits and sex determination in ninespine sticklebacks mapped to different regions than did the major loci controlling the corresponding traits in threespine sticklebacks. The convergent evolution of changes in skin color and the number of lateral plates in different threespine stickleback populations has often taken place by repeated selection of ancient variants of the *Kitlg* and *Eda* genes, respectively [8, 10]. These variants are present at low levels in migratory marine populations and were presumably introduced into new locations when marine ancestors colonized new lakes and streams. Perhaps recent evolution from standing variation within a single species of stickleback is more likely to involve the same genes in different populations, whereas convergent evolution between more distantly related genera may be more likely to arise from independent mutations. The current study suggests that ninespine sticklebacks provide an outstanding system for finding additional genes responsible for morphological diversity in natural populations of vertebrates and comparing the detailed genetic basis of convergent evolutionary change in long-separated lineages.

Supplemental Data

Supplemental data include Supplemental Experimental Procedures, two figures, and five tables and can be found at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01130-0.

Acknowledgments

We thank Frank von Hippel and the Department of Biological Sciences at the University of Alaska, Anchorage for field assistance and laboratory facilities; the Broad Institute for the initial release of the threespine stickleback genome assembly; Katie Peichel, Craig Miller, and three anonymous reviewers for comments on the manuscript; and Frank Chan, Pam Colosimo, Jonathan Craft, Katie Ellis, Tiffani Jones, Melissa Marks, and Sydney Stringham for experimental assistance. This work was supported by the Helen Hay Whitney Foundation, a Burroughs Wellcome Career Award in the Biomedical Sciences, and National Science Foundation (NSF) grant IOS-0744974 (M.D.S.); an NSF Graduate Research Fellowship (B.R.S.); the University of Utah Biology Undergraduate Research Program (A.L.M.); NSF grants DEB-0211391 and DEB-0322818 (M.A.B. and F.J.Rohlf); and National Institutes of Health Center of Excellence in Genomic Science grant 1P50HG02568 (D.M.K.). D.M.K. is an Investigator of the Howard Hughes Medical Institute.

Received: November 25, 2008
Revised: April 10, 2009
Accepted: May 11, 2009
Published online: June 4, 2009

References

1. Arendt, J., and Reznick, D. (2008). Convergence and parallelism reconsidered: What have we learned about the genetics of adaptation? Trends Ecol. Evol. *23*, 26–32.
2. Gould, S.J. (2002). The Structure of Evolutionary Theory (Cambridge, MA: The Belknap Press of Harvard University Press).
3. Peichel, C.L., Nereng, K., Ohgi, K.A., Cole, B.L.E., Colosimo, P.F., Buerkle, C.A., Schluter, D., and Kingsley, D.M. (2001). The genetic architecture of divergence between threespine stickleback species. Nature *414*, 901–905.
4. Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jonsson, B., Schluter, D., and Kingsley, D.M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature *428*, 717–723.
5. Colosimo, P.F., Peichel, C.L., Nereng, K., Blackman, B.K., Shapiro, M.D., Schluter, D., and Kingsley, D.M. (2004). The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol. *2*, e109.
6. Cresko, W.A., Amores, A., Wilson, C., Murphy, J., Currey, M., Phillips, P., Bell, M.A., Kimmel, C.B., and Postlethwait, J.H. (2004). Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc. Natl. Acad. Sci. USA *101*, 6050–6055.
7. Peichel, C.L., Ross, J.A., Matson, C.K., Dickson, M., Grimwood, J., Schmutz, J., Myers, R.M., Mori, S., Schluter, D., and Kingsley, D.M. (2004). The master sex-determination locus in threespine sticklebacks is on a nascent y chromosome. Curr. Biol. *14*, 1416–1424.
8. Colosimo, P.F., Hosemann, K.E., Balabhadra, S., Villarreal, G., Jr., Dickson, M., Grimwood, J., Schmutz, J., Myers, R.M., Schluter, D., and Kingsley, D.M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science *307*, 1928–1933.
9. Kimmel, C.B., Ullmann, B., Walker, C., Wilson, C., Currey, M., Phillips, P.C., Bell, M.A., Postlethwait, J.H., and Cresko, W.A. (2005). Evolution and development of facial bone morphology in threespine sticklebacks. Proc. Natl. Acad. Sci. USA *102*, 5791–5796.
10. Miller, C.T., Beleza, S., Pollen, A.A., Schluter, D., Kittles, R.A., Shriver, M.D., and Kingsley, D.M. (2007). cis-regulatory changes in Kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. Cell *131*, 1179–1189.
11. Cole, N.J., Tanaka, M., Prescott, A., and Tickle, C.A. (2003). Expression of limb initiation genes and clues to the basis of morphological diversification in threespine sticklebacks. Curr. Biol. *13*, R951–R952.
12. Coyle, S.M., Huntingford, F.A., and Peichel, C.L. (2007). Parallel evolution of *Pitx1* underlies pelvic reduction in Scottish three-spined stickleback (*Gasterosteus aculeatus*). J. Hered. *98*, 581–586.
13. Bell, M.A., Stewart, J.D., and Park, P.J. (2009). The world's oldest fossil threespine stickleback fish. Copeia *2009*, 256–265.
14. Nelson, J.S. (1971). Absence of the 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.
15. Bell, M.A., Orti, G., Walker, J.A., and Koenings, J.P. (1993). Evolution of pelvic reduction in threespine stickleback fish: a test of competing hypotheses. Evol. Int. J. Org. Evol. *47*, 906–914.
16. Chen, T.-R., and Reisman, H.M. (1970). A comparative chromosome study of the North American species of sticklebacks (Teleostei: Gasterosteidae). Cytogenetics *9*, 321–332.
17. Ocalewicz, K., Fopp-Bay at, D.F., Woznicki, P., and Jankun, M. (2008). Heteromorphic sex chromosomes in the ninespine stickleback *Pungitius pungitius*. J. Fish Biol. *73*, 456–462.
18. Ross, J.A., Urton, J.R., Boland, J., Shapiro, M.D., and Peichel, C.L. (2009). Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). PLoS Genet *5*, e1000391.
19. Hunt, G., Bell, M.A., and Travis, M.P. (2008). Evolution toward a new adaptive optimum: Phenotypic evolution in a fossil stickleback lineage. Evol. Int. J. Org. Evol. *62*, 700–710.
20. Blouw, D.M., and Boyd, G.J. (1992). Inheritance of reduction, loss, and asymmetry of the pelvis in *Pungitius pungitius* (ninespine stickleback). Heredity *68*, 33–42.
21. Giles, N. (1983). The possible role of environmental calcium levels during the evolution of phenotypic diversity on Outer Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. J. Zool. *4*, 535–544.
22. Reimchen, T.E. (1980). Spine deficiency and polymorphism in a population of *Gasterosteus aculeatus*: An adaptation to predators? Can. J. Zool. *58*, 1232–1244.
23. Ziuganov, V.V., and Zotin, A.A. (1995). Pelvic girdle polymorphism and reproductive barriers in the ninespine stickleback *Pungitius pungitius* (L.) from northwest Russia. Behaviour *132*, 1095–1105.

24. Zyuganov, V.V., and Rosanov, A.S. (1987). Genetics of pelvic girdle reduction in fish (as illustrated by the ninespine stickleback *Pungitius pungitius* L.). Doklady Akademii Nauk SSR *293*, 155–159.
25. Marchinko, K.B. (2009). Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. Evol. Int. J. Org. Evol. *63*, 127–138.
26. Shapiro, M.D., Marks, M.E., Peichel, C.L., Nereng, K., Blackman, B.K., Jonsson, B., Schluter, D., and Kingsley, D.M. (2006). Corrigendum: Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature *439*, 1014.
27. Szeto, D.P., Rodriguez-Esteban, C., Ryan, A.K., O'Connell, S.M., Liu, F., Kioussi, C., Gleiberman, A.S., Izpisúa-Belmonte, J.C., and Rosenfeld, M.G. (1999). Role of the Bicoid-related homeodomain factor *Pitx1* in specifying hindlimb morphogenesis and pituitary development. Genes Dev. *13*, 484–494.
28. Logan, M., and Tabin, C.J. (1999). Role of *Pitx1* upstream of *Tbx4* in specification of hindlimb identity. Science *283*, 1736–1739.
29. Shang, J., Luo, Y., and Clayton, D.A. (1997). Backfoot is a novel homeobox gene expressed in the mesenchyme of developing hind limb. Dev. Dyn. *209*, 242–253.
30. Shapiro, M.D., Bell, M.A., and Kingsley, D.M. (2006). Parallel genetic origins of pelvic reduction in vertebrates. Proc. Natl. Acad. Sci. USA *103*, 13753–13758.
31. Nelson, J.S. (1971). Comparison of the pectoral and pelvic skeletons and of some other bones and their phylogenetic implications in the Aulorhynchidae and Gasterosteidae (Pisces). J. Fish. Res. Board Can. *28*, 427–442.
32. Bell, M.A., Khalef, V., and Travis, M.P. (2007). Directional asymmetry of pelvic vestiges in threespine stickleback. J. Exp. Zoolog. B Mol. Dev. Evol. *308*, 189–199.
33. Marcil, A., Dumontier, E., Chamberland, M., Camper, S.A., and Drouin, J. (2003). *Pitx1* and *Pitx2* are required for development of hindlimb buds. Development *130*, 45–55.
34. Gibson, G., and Dworkin, I. (2004). Uncovering cryptic genetic variation. Nat. Rev. Genet. *5*, 681–690.
35. Devlin, R.H., and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture *208*, 191–364.
36. Mank, J.E., Promislow, D.E., and Avise, J.C. (2006). Evolution of alternative sex-determining mechanisms in teleost fishes. Biol. J. Linn. Soc. Lond. *87*, 83–93.
37. Woram, R.A., Gharbi, K., Sakamoto, T., Hoyheim, B., Holm, L.E., Naish, K., McGowan, C., Ferguson, M.M., Phillips, R.B., Stein, J., et al. (2003). Comparative genome analysis of the primary sex-determining locus in salmonid fishes. Genome Res. *13*, 272–280.
38. Chae, B.S., and Yang, H.J. (1990). Sexual dimorphism in eightspine stickleback, *Pungitius sinensis*: Gasterosteidae. Korean Journal of Zoology *33*, 260–265.
39. Kobayashi, H. (1959). Cross-experiments with three species of stickleback, *Pungitius pungitius* (L.), *Pungitius tymensis* (Nikolsk), and *Pungitius sinensis* (Guichenot), with special reference to their systematic relationship. Journal of Hokkaido Gakugei University. Section B *10*, 363–384.
40. Kitano, J., Mori, S., and Peichel, C.L. (2007). Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). Copeia *2007*, 336–349.
41. Caldecutt, W.J., and Adams, D.C. (1998). Morphometrics of trophic osteology in the threespine stickleback, *Gasterosteus aculeatus*. Copeia *1998*, 827–838.
42. Caldecutt, W.J., Bell, M.A., and Buckland-Nicks, J.A. (2001). Sexual dimorphism and geographic variation in dentition of threespine stickleback, *Gasterosteus aculeatus*. Copeia *2001*, 936–944.
43. McPhail, J.D. (1992). 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.
44. Albert, A.Y., Sawaya, S., Vines, T.H., Knecht, A.K., Miller, C.T., Summers, B.R., Balabhadra, S., Kingsley, D.M., and Schluter, D. (2008). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. Evol. Int. J. Org. Evol. *62*, 76–85.
45. Aguirre, W.E., Ellis, K.E., Kusenda, M., and Bell, M.A. (2008). Phenotypic variation and sexual dimorphism in anadromous threespine stickleback: Implications for postglacial adaptive radiation. Biol. J. Linn. Soc. Lond. *95*, 465–478.
46. Charlesworth, D., Charlesworth, B., and Marais, G. (2005). Steps in the evolution of heteromorphic sex chromosomes. Heredity *95*, 118–128.
47. Sucena, E., Delon, I., Jones, I., Payre, F., and Stern, D.L. (2003). Regulatory evolution of *shavenbaby/ovo* underlies multiple cases of morphological parallelism. Nature *424*, 935–938.
48. Gompel, N., Prud'homme, B., Wittkopp, P.J., Kassner, V.A., and Carroll, S.B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in *Drosophila*. Nature *433*, 481–487.
49. Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.D., True, J.R., and Carroll, S.B. (2006). Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. Nature *440*, 1050–1053.
50. Davis, G.K., Srinivasan, D.G., Wittkopp, P.J., and Stern, D.L. (2007). The function and regulation of *Ultrabithorax* in the legs of *Drosophila melanogaster*. Dev. Biol. *308*, 621–631.
51. Hoekstra, H.E. (2006). Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity *97*, 222–234.
52. Protas, M., Conrad, M., Gross, J.B., Tabin, C., and Borowsky, R. (2007). Regressive evolution in the Mexican cave tetra, *Astyanax mexicanus*. Curr. Biol. *17*, 452–454.
53. Wilkens, H. (1971). Genetic interpretation of regressive evolutionary process: Studies of hybrid eyes of two *Astyanax* cave populations (Characidae, Pisces). Evol. Int. J. Org. Evol. *25*, 530–544.
54. Borowsky, R. (2008). Restoring sight in blind cavefish. Curr. Biol. *18*, R23–R24.
55. Hoekstra, H.E., Hirschmann, R.J., Bundey, R.A., Insel, P.A., and Crossland, J.P. (2006). A single amino acid mutation contributes to adaptive beach mouse color pattern. Science *313*, 101–104.
56. Steiner, C.C., Rompler, H., Boettger, L.M., Schoneberg, T., and Hoekstra, H.E. (2009). The genetic basis of phenotypic convergence in beach mice: Similar pigment patterns but different genes. Mol. Biol. Evol. *26*, 35–45.
57. Hoekstra, H.E., and Nachman, M.W. (2003). Different genes underlie adaptive melanism in different populations of rock pocket mice. Mol. Ecol. *12*, 1185–1194.
58. Wittkopp, P.J., Williams, B.L., Selegue, J.E., and Carroll, S.B. (2003). *Drosophila* pigmentation evolution: Divergent genotypes underlying convergent phenotypes. Proc. Natl. Acad. Sci. USA *100*, 1808–1813.
59. van Ooijen, J.W. (1999). LOD significance thresholds for QTL analysis in experimental populations of diploid species. Heredity *83*, 613–624.