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Authors

Cleves, Phillip A
Ellis, Nicholas A
Jimenez, Monica T
et al.

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Evolved tooth gain in sticklebacks is associated with a *cis*-regulatory allele of *Bmp6*

Phillip A. Cleves^a, Nicholas A. Ellis^a, Monica T. Jimenez^a, Stephanie M. Nunez^{b,c,1}, Dolph Schluter^d, David M. Kingsley^{b,c}, and Craig T. Miller^{a,2}

^aDepartment of Molecular and Cell Biology, University of California, Berkeley, CA 94720; ^bDepartment of Developmental Biology and ^cHoward Hughes Medical Institute, Stanford University, Stanford, CA 94305; and ^dDepartment of Zoology, University of British Columbia, Vancouver, Canada V6T 1Z4

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Developmental genetic studies of evolved differences in morphology have led to the hypothesis that *cis*-regulatory changes often underlie morphological evolution. However, because most of these studies focus on evolved loss of traits, the genetic architecture and possible association with *cis*-regulatory changes of gain traits are less understood. Here we show that a derived benthic freshwater stickleback population has evolved an approximate twofold gain in ventral pharyngeal tooth number compared with their ancestral marine counterparts. Comparing laboratory-reared developmental time courses of a low-toothed marine population and this high-toothed benthic population reveals that increases in tooth number and tooth plate area and decreases in tooth spacing arise at late juvenile stages. Genome-wide linkage mapping identifies largely separate sets of quantitative trait loci affecting different aspects of dental patterning. One large-effect quantitative trait locus controlling tooth number fine-maps to a genomic region containing an excellent candidate gene, *Bone morphogenetic protein 6* (*Bmp6*). Stickleback *Bmp6* is expressed in developing teeth, and no coding changes are found between the high- and low-toothed populations. However, quantitative allele-specific expression assays of *Bmp6* in developing teeth in F1 hybrids show that *cis*-regulatory changes have elevated the relative expression level of the freshwater benthic *Bmp6* allele at late, but not early, stages of stickleback development. Collectively, our data support a model where a late-acting *cis*-regulatory up-regulation of *Bmp6* expression underlies a significant increase in tooth number in derived benthic sticklebacks.

Gasterosteus | polyphyodonty | craniofacial | adaptation | quantitative genetics

Understanding the developmental genetic basis of morphological evolution is a long-standing goal in biology (1, 2). Evolved morphological differences can be “loss” (regressive) traits, where morphological features are lost or reduced, or “gain” (constructive) traits, where morphological features are gained or increased. Although many of the traits best understood at the molecular level involve loss traits (1, 2), recent studies have begun to genetically dissect some evolved gain traits (3–5). However, whether gain traits have similar genetic architectures as loss traits and whether gain traits are also associated with *cis*-regulatory changes remains largely unknown.

Teeth are a classic vertebrate model system for studying morphological evolution, due to their excellent preservation in the fossil record. Teeth are also a classic vertebrate model system for organogenesis, because teeth, like many other organs, develop through reciprocal signaling interactions between epithelia and mesenchyme. Continuing efforts have produced a rich understanding of the genetic networks that orchestrate tooth morphogenesis in model systems (6). However, despite the wealth of knowledge about tooth evolution and development, we still know little about the number and type of genetic changes that accompany diversification of dental patterning during evolution.

Pharyngeal jaws and teeth, used during mastication in fish, are located in the posterior branchial segments in the fish’s throat (7, 8). In teleost fish, pharyngeal jaw patterning is an adaptive trait

that covaries with diet and trophic niche (9). The rich phenotypic diversity of pharyngeal jaws and teeth in fish, coupled with the understanding of the genetic networks of tooth development from model organisms, offers an opportunity to understand the developmental genetic basis of evolved changes in tooth patterning.

The threespine stickleback (*Gasterosteus aculeatus*) fish has emerged as an excellent model system allowing for genetic dissection of evolutionary change in vertebrates (10). Sticklebacks have undergone an extensive adaptive radiation, independently colonizing thousands of freshwater lakes and creeks generated after widespread melting of glaciers at the end of the last ice age (11). The dietary shifts to larger prey accompanying freshwater adaptation have resulted in evolved changes in trophic morphology (12, 13). Despite striking morphological differences between marine and freshwater populations, hybrids are fertile, allowing forward genetic analysis of evolved differences. In several lakes, “species pairs” of benthic and limnetic stickleback morphs are found (13). In each of these lakes, a benthic species is adapted to feeding on macroinvertebrates in the littoral zone or deeper sediments. This derived diet differs from the diet of both the limnetic species and ancestral marine forms, both of which feed on smaller zooplankton. Benthic sticklebacks have evolved trophic adaptations matched for this specialized diet (13, 14). Here we describe evolved tooth gain, a heritable constructive increase in tooth number compared with ancestral marine

Significance

How body pattern evolves in nature remains largely unknown. Although recent progress has been made on the molecular basis of losing morphological features during adaptation to new environments (regressive evolution), there are few well worked out examples of how morphological features may be gained in natural species (constructive evolution). Here we use genetic crosses to study how threespine stickleback fish have increased their tooth number in a new freshwater environment. Genetic mapping and gene expression experiments suggest regulatory changes have occurred in the gene for a bone morphogenetic signaling molecule, leading to increased expression in the freshwater fish that have more teeth. Our studies suggest that changes in gene regulation may underlie both gain and loss traits during vertebrate evolution.

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¹Present address: University of Michigan School of Dentistry, Ann Arbor, MI 48109.

²To whom correspondence should be addressed. Email: ctmiller@berkeley.edu.

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of the seven tooth-patterning QTL, only three go in a direction that is concordant with the overall shift in tooth number in the parental populations (i.e., benthic alleles conferring more teeth) based on the developmental time courses. However, the QTL with the largest phenotypic effect on chromosome 21 does act in a direction that is consistent with the overall trend in tooth number in the parental populations (benthic allele conferring more teeth). Perhaps the smaller-effect QTL that have effects in the opposite direction result from chromosome 21's effect overshooting the adaptive peak for tooth patterning in this recently evolved population, with other loci evolving to bring tooth patterning closer to the adaptive peak (34). The mixed direction of effects of benthic alleles could alternatively result from pleiotropy (35), with QTL controlling other adaptive benthic phenotypes that might secondarily affect tooth patterning. For example, the large-effect tooth-spacing QTL on chromosome 4 overlaps the *Ectodysplasin* (*Eda*) gene which controls adaptive reductions in armor plate patterning (36) and is also well known to affect vertebrate tooth patterning (37, 38). Interestingly, *Eda* also plays a role in the spacing of hair placodes and tooth cusps in mice (39, 40), making *Eda* an excellent candidate for underlying the tooth-spacing QTL on chromosome 4. A third possibility is that some or all of these tooth traits could be changing due to genetic drift occurring after freshwater colonization. As several other species pairs and hundreds of other freshwater populations with trophic modifications have been described (12, 13, 41–43), one test of adaptive significance will be to ask whether other derived benthic lake or creek freshwater stickleback populations have also evolved increases in tooth number. Molecular genetic identification of the tooth-patterning QTL that are segregating in the current cross, combined with population genetic tests of molecular variation surrounding causal loci, should also help distinguish these models.

To begin to study the molecular mechanisms behind evolved tooth gain, we fine-mapped the largest-effect tooth number QTL on chromosome 21. A previous study identified a cluster of QTL on chromosome 21 controlling several derived freshwater skeletal traits (15). This QTL cluster mapped near a large genomic inversion previously shown to display strong worldwide patterns of divergence between marine and freshwater populations (44), suggesting that multiple phenotypes may be controlled by linked genetic changes within the chromosome inversion. Interestingly, we find that the 1.5-LOD candidate interval for the chromosome 21 tooth QTL maps over 1.5 Mb from the inversion, strongly suggesting that the molecular changes driving tooth gain map outside the inverted region.

The new fine-mapped interval for the tooth QTL contains an excellent candidate gene, *Bmp6*. We show that *Bmp6* is expressed in developing teeth in marine and benthic sticklebacks, has no predicted coding changes between populations, but has a late-onset *cis*-regulatory up-regulation in benthic fish. Because in other vertebrates, BMPs act as activators of tooth development (45), we hypothesize that the elevated *Bmp6* expression observed in benthic sticklebacks contributes to their increased tooth number controlled by the chromosome 21 region. Bone Morphogenetic Proteins were originally identified based on their remarkable ability to induce ectopic bone when implanted at new sites in animals (46). Thus, increases in tooth plate area could also result from increased *Bmp6* expression. The divergence in tooth number and *Bmp6* *cis*-regulation at late, not early, developmental stages might reflect a heterochronic shift in the benthic population, where the benthic tooth development and replacement program is “stuck” in the early rapid tooth-generating phase observed in early larval stages in both marine and benthic fish. Although we parsimoniously favor the hypothesis that *Bmp6* underlies the evolved differences in tooth number, tooth plate area, and intertooth spacing, we note that the fine mapping was only done for tooth number, so it is possible that other genes underlie the evolved changes in tooth plate area and intertooth spacing.

The use of BMP ligands as major drivers of morphological evolution in vertebrates is striking. BMP family members have been implicated in several vertebrate evolved traits: size and shape of the beak in Darwin's finches, size and shape of the jaw in cichlids, jaw and skull variation in brachycephalic dogs, and avian feather patterning (47–50). Although based on a limited number of reported cases and possibly affected by ascertainment biases, this apparent reuse of the same signaling pathway across taxa may reflect a predisposition for *Bmp* genes to be used during morphological evolution, perhaps due to having complex, modular *cis*-regulatory architecture to generate evolutionary variation (51, 52).

Previous QTL mapping studies in sticklebacks have shown that major changes in pelvic hindfin development, armor plate formation, and body pigmentation are all due to alterations in key developmental signaling molecules and transcription factors (36, 53–55). In each of these previous cases, freshwater fish have evolved a major loss or reduction of skeletal structures that were originally present in marine ancestors. In all three cases, *cis*-regulatory changes are implicated, either directly (53, 54) or inferred (36). Here we show that a major gain in tooth number can also be genetically mapped to a relatively small number of chromosome regions. The QTLs with largest effects on tooth number control somewhat less of the overall variance than the previously identified QTL for armor plates, pelvis, and pigment (each of which controls 50% or more of the variance in the corresponding trait). Nevertheless, the overall effects of the tooth-patterning QTLs are still quite large compared with classical predictions of nearly infinitesimal effects for genetic changes underlying evolved differences in natural populations. Finally, our results with *Bmp6* show that for both loss and gain traits, the chromosome regions with largest phenotypic effects show clear evidence of *cis*-acting regulatory changes in key developmental control genes. Although many more case studies will be needed to draw general conclusions, collectively, these studies suggest that similar general principles may underlie the evolution of both loss and gain traits and that regulatory changes in developmental control genes play an important role in both regressive and constructive evolution of the vertebrate skeleton.

Materials and Methods

Stickleback Husbandry. Lab-reared fish were raised in 110-liter tanks under common conditions (3.5 g/l Instant Ocean salt, 0.4 mL/l NaHCO₃) and fed live brine shrimp as larvae, then frozen daphnia, bloodworms, and Mysis shrimp as juveniles and adults. All experiments and field collections were done with the approval of the Institutional Animal Care and Use Committee from University of California, Berkeley, Stanford University, or the University of British Columbia.

QTL Mapping. QTL mapping was done using R/qtl (56). To map QTL for adult tooth number, area, and spacing, we analyzed a subset ($n = 272$ fish) of a previously described (16) Paxton Benthic and Japanese Marine F2 cross. Two hundred seventy-five microsatellite markers were genotyped in each F2. Tooth number, area, and spacing were quantified in each F2. As all three traits were significantly correlated with fish total length, residuals from a linear regression were used for each of the three traits. See *SI Appendix, SI Materials and Methods*, for details of QTL mapping.

In Situ Hybridization. Marine and benthic embryos and larvae were euthanized, fixed overnight in 4 g paraformaldehyde in 100 ml 1×PBS, then dehydrated and stored at -20°C in methanol. For larvae older than 9 d postfertilization, ventral tooth plates were dissected after rehydration from methanol and before in situ hybridization. In situ hybridization was performed essentially as described (57) but with in situ done in tubes in a water bath not baskets and using a 2-d hybridization for older larval stages. For sections, whole-mount in situ were fixed overnight in 4 g paraformaldehyde in 100 ml 1×PBS, embedded in gelatin-albumin cross-linked with 1.75% glutaraldehyde, and sectioned at 40 μm on a Pelco 101 Vibratome Series 1000. Primer sequences for generating the clones used to make the *Bmp6*, *Shh*, *Pitx2*, and *Tfap2a* riboprobes are listed in *SI Appendix*.

Pyrosequencing of F1 Hybrids. For allele-specific expression experiments, Paxton benthic freshwater fish were crossed with Rabbit Slough marine fish by in vitro fertilization to generate hybrid F1s. The bilateral pair of ventral pharyngeal tooth plates from each hybrid was dissected on ice from larval, juvenile, and adult stages (~10–20 mm, $n = 12$; ~25–40 mm, $n = 18$; and >40 mm in total length, $n = 13$, respectively). See *SI Appendix* for primer sequences used for RT-PCR and pyrosequencing and additional methods.

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