Widespread Parallel Evolution in Sticklebacks by Repeated Fixation of Ectodysplasin Alleles

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Major phenotypic changes evolve in parallel in nature by molecular mechanisms that are largely unknown. Here, we use positional cloning methods to identify the major chromosome locus controlling armor plate patterning in wild threespine sticklebacks. Mapping, sequencing, and transgenic studies show that the Ectodysplasin (EDA) signaling pathway plays a key role in evolutionary change in natural populations and that parallel evolution of stickleback low-plated phenotypes at most freshwater locations around the world has occurred by repeated selection of *Eda* alleles derived from an ancestral low-plated haplotype that first appeared more than two million years ago. Members of this clade of low-plated alleles are present at low frequencies in marine fish, which suggests that standing genetic variation can provide a molecular basis for rapid, parallel evolution of dramatic phenotypic change in nature.

Particular phenotypic traits often evolve repeatedly when independent populations are exposed to similar ecological conditions (*1*, *2*). The threespine stickleback species complex (*Gasterosteus aculeatus*) provides an ideal system for further studying the molecular mechanisms that underlie widespread parallel evolution of phenotypic traits in nature. Parallel evolution within this species complex has occurred in countless freshwater lake and stream environments colonized by marine sticklebacks after widespread melting of glaciers 10,000 to 20,000 years ago. The young age of the freshwater populations, the ability to generate fertile hybrids between divergent populations, and the recent development of stickleback genomic resources make it possible to map the genes that control evolutionary change and to compare the genetic basis of similar traits that have evolved in different locations (*3*–*7*).

Several marine phenotypes have changed repeatedly in new freshwater environments, which includes reduction of the extensive bony armor found in ocean fish (*8*, *9*). Marine sticklebacks typically have a continuous row of 32 to 36 armor plates extending from head to tail ("complete" morph, Fig. 1A). In contrast, innumerable freshwater populations have a gap in the middle of the row of plates ("partial" morph), or retain only zero to nine plates at the anterior end ("low" morph, Fig. 1A) (*10*, *11*). Several factors have been proposed that could contribute to the selective advantage of armor plate reduction after colonization of new lakes and streams by completely plated marine ancestors, including low calcium levels, increased body flexibility and maneuverability, changes in swimming performance, and changes in predation regimes in freshwater environments (*12*–*17*).

Positional cloning of the plate morph region. Further understanding of the molecular basis of parallel evolution requires the identification of the genes and mutations that underlie major phenotypic change. We previously used genome-wide linkage mapping in a marine by freshwater F_2 cross to show that armor plate patterns are controlled primarily by a single major QTL that maps to linkage group IV and by four minor modifier QTL that cause smaller quantitative variation in the size and number of plates (*4*). To refine the position of the major locus, we screened for new amplified fragment-length polymorphism markers (*18*) that differed in allele size or frequency in pools of low and completely plated progeny from a marine by freshwater cross. Newly isolated markers *Stn345* and *Stn346* were much more closely linked than previous markers, defining a new interval of 0.68 cM for the plate morph locus (Fig. 1B).

Stn345 and *Stn346* were used to screen a bacterial artificial chromosome (BAC) library derived from completely plated marine fish from Salmon River, Canada (SRMA) (*7*). Three rounds of chromosome walking led to the isolation of six overlapping BAC clones spanning the interval of interest (Fig. 1B). In a total of 1166 chromosomes, *Stn345* and BAC end marker *Stn347* mapped two recombination events proximal and one recombination event distal of the plate morph locus, respectively. These recombination events define a physical region of about 539 kb that must contain the plate morph locus. BAC clones A and B (Fig. 1B) were completely sequenced during the chromosome walk, generating a contiguous sequence assembly of 407,051 base pairs (bp) derived from the plate morph region.

Linkage disequilibrium screening. Meioses and interbreeding in natural populations tend to homogenize allele frequencies at most loci. However, particular alleles that are very closely linked to a mutation of interest may remain in linkage disequilibrium with that mutation for many generations (*19*). To test for possible linkage disequilibrium in the region controlling plate morph phenotypes, we designed microsatellite markers at intervals of ~12 kb throughout the BAC sequence assembly and examined the distribution of alleles at each marker in a sample of 46 completely and 45 low-plated fish from a single interbreeding wild population from Friant, CA (FRI) (*4*, *20*). The difference in allele distribution at *Stn365* in completely and low-plated fish was 10^7 times more significant than the differences at flanking markers *Stn364* and *Stn366*, which define a region of peak linkage disequilibrium of ~16 kb (Fig. 1C).

Gene predictions show that the marker at the peak of linkage disequilibrium is located within intron 2 of the stickleback *Ectodysplasin* (*Eda*) gene. EDA is a member of the tumor necrosis family of secreted signaling molecules and, in mammals, is required for proper development of a number of ectodermal derivatives (e.g., teeth, hair, and sweat glands) and dermal bones (*21*, *22*). Previous studies have shown that a mutation in the *Ectodysplasin receptor* (*Edar*) gene in medaka (*Oryzias latipes*) causes loss of most scales (*23*), which are elements of the dermal skeleton (*24*). Many elements of the dermal skeleton in fishes, including scales and the dermal lateral plates of sticklebacks, have likely evolved from a common ancestral element (*24*). The position of *Eda* at the peak of linkage disequilibrium in the stickleback candidate interval and the known role of EDA signaling in scale formation suggested that changes in the *Eda* locus may underlie the molecular basis of plate morph evolution in sticklebacks.

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***Eda* structure in low and complete morphs.** To compare the structure of *Eda* and linked genes in marine and freshwater sticklebacks, we isolated two overlapping BAC clones from a library derived from the low-plated benthic Paxton Lake, Canada population (PAXB) (*7*), and completely sequenced the *Eda* region. Comparison to the sequence of the completely plated SRMA marine population identified numerous noncoding changes (table S2), and four mutations that lead to amino acid changes in EDA protein (Fig. 2). None of the amino acid changes occur at sites that are highly conserved between mammals and fish or in residues that have previously been associated with defects in humans (*25*, *26*). Three of the four sites also vary among other fish species.

Previous studies in mammals have shown that *Eda* undergoes alternative splicing to produce two protein isoforms that differ in length by two amino acids and bind different receptors (*27*, *28*). Both splicing isoforms were recovered from developing low- and completely plated fish (10- to 20-mm standard length) with the use of reverse transcription polymerase chain reaction (RT-PCR), and no other splicing changes were seen in the low-plated fish.

We also generated probes to examine the spatial pattern of expression of *Eda* in sticklebacks, but we were unable to detect significant *Eda* expression in any samples using whole-mount in situ hybridization, even at stages where we could recover spliced *Eda* mRNA using RT-PCR. Difficulties in detecting *Eda* expression by in situ hybridization have also been reported in several other organisms, presumably because of low levels of expression during normal development (*29*, *30*).

Molecular basis of parallel evolution. Because previous complementation and genetic mapping results suggest that the same major locus controls lateral plate patterning in multiple freshwater populations around the world (*2*, *4*, *6*), we expanded the survey of *Eda* sequence to 10 completely plated marine populations and 15 low-plated populations collected from sites throughout the Northern Hemisphere (Fig. 3A, table S1). It was interesting that most of the low-plated populations, although from diverse regions, shared many of the base-pair changes previously seen in the PAXB population. One clear exception was a low-morph population from Nakagawa Creek, Japan (NAKA), which had no mutations that would alter the EDA amino acid sequence found in marine sticklebacks (SRMA) (Fig. 2). Previous genetic crosses show that low-plated fish from the NAKA population fail to complement the low-plated phenotype of fish from Salmon River, British Columbia (SRST) (*2*), a population that does share the characteristic *Eda* sequence changes seen in PAXB and most other low-plated populations. These data suggest that the low-plated phenotype in NAKA is due to an independently derived allele of *Eda*.

To further analyze the history of sequence changes in the plate morph region, we built phylogenetic trees using 1328 bp of exon and intron sequence from the *Eda* locus (Fig. 3B). Bayesian, maximum parsimony, and maximum likelihood trees all showed that *Eda* sequences from every low-plated population except NAKA belonged to a distinct clade (posterior probability of 1.00 in MrBayes trees and bootstrap support of 99.7 and 100% in parsimony and maximum likelihood trees, respectively). These results show that *Eda* alleles of most low-plated populations share a common ancestry. The exception is the low-plated allele within the NAKA population, which clearly has a separate origin.

Despite the shared ancestry of *Eda* alleles, it is highly unlikely that an ancestral population of low-plated fish migrated through the ocean to found most other low-plated populations in freshwater lakes and streams around the world. Ocean sticklebacks are virtually always completely plated. In addition, previous phylogenetic analysis of mitochondrial sequences reject the possibility that low-plated fish have a single origin (*9*, *31*, *32*). To further test whether low-plated populations have multiple origins, we amplified sequences from 25 random nuclear genes and scored single-nucleotide polymorphisms (SNPs) at 193 sites from 20 different completely and low-plated populations. Trees built from the nuclear sequences showed no evidence for a single origin of low-plated populations (Fig. 3C). The

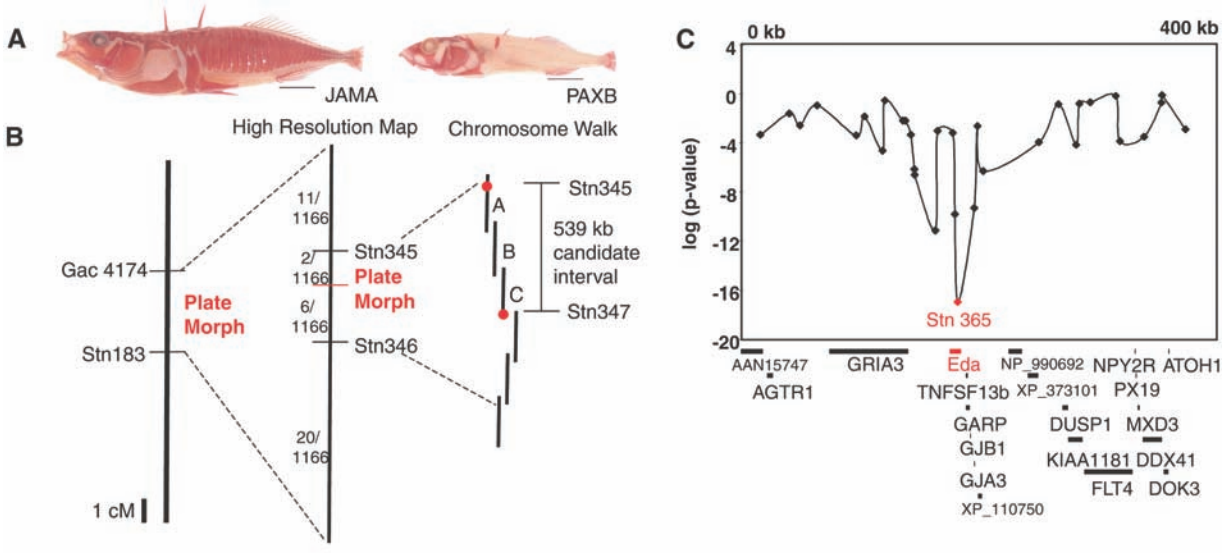

Fig. 1. Genetic, physical, and linkage disequilibrium map of the plate morph interval. (**A**) Complete and low-morph phenotypes in alizarin red–stained specimens (*4*) of Japanese Marine (JAMA, left) and benthic Paxton Lake (PAXB, right) sticklebacks, the parent populations of a large F_2 mapping cross. Other skeletal changes evident in PAXB fish, such as reduction of pelvis and spines, map to different chromosomes (*5*). Scale bars, 1 cm. (**B**) High-resolution genetic and physical mapping. Newly isolated markers *Stn345* and *Stn346* rarely recombine with the plate morph locus (numbers of recombinants in 1166 chromosomes shown). Six overlapping BAC clones span the genetic interval, and markers *Stn345* and *Stn347* (red dots) map to opposite sides of the plate morph locus. (**C**) Linkage disequilibrium screening. Two sequenced BAC clones were used to develop new microsatellite markers (*Stn348*–*Stn379*) located 15, 39, 48, 63, 96, 103, 118, 120, 135, 137, 142, 145, 145, 163, 165, 177, 179, 182, 195, 198, 203, 250, 267, 282, 284, 293, 315, 319, 339, 353, 354, and 374 kilobases from *Stn345* (black dots). *Stn365*, located in the stickleback *Eda* locus, showed large differences in allele frequency in completely and low-plated fish from Friant, CA. Positions of other genes in the sequenced interval are shown, with human genome nomenclature committee (HGNC) designations, or accession numbers of the best matches in National Center for Biotechnology Information (NCBI) BLAST searches.

best-supported branches were related by geography rather than plate phenotype. For example, a clade from the Atlantic ocean contained both completely and low-plated populations (posterior probability of 1.00 in MrBayes trees and bootstrap support of 68 and 54% in maximum likelihood and parsimony trees, respectively). The topologies of EDA and nuclear sequence trees for identical populations were significantly different, whether trees were constructed using Bayesian and maximum likelihood methods (Kishino-Hasegawa tests: $P < 0.0005$), or maximum parsimony approaches (Wilcoxon signed rank test: $P < 0.0001$). Most important, the nuclear data firmly reject the specific hypothesis of monophyly for all low-plated populations except NAKA (Kishino-Hasegawa test on maximum likelihood trees: $P < 0.0005$, Wilcoxon test on parsimony trees: $P < 0.0015$). On the basis of these and the previous mitochondrial studies, current low-plated populations are clearly not derived from a single low-plated population that has colonized different environments.

The most likely interpretation to account for the global sharing of closely related low-plated alleles at the *Eda* locus is that the alleles controlling the low-plated phenotype are present at some frequency in marine populations. To test this, we collected large samples of completely plated fish at the ocean outlets of the Navarro River in California (NAV) and the Little Campbell River in British Columbia (LITC). We typed DNA samples from all individuals with two markers that are located within introns 2 and 6 of *Eda* and that distinguish complete and low-morph alleles found in most populations (*Stn380* and *Stn381*). Eight of 109 completely plated fish from NAV were heterozygous for complete and low-morph alleles at both markers, which gave an estimated frequency of low-morph alleles of 3.8% (8/218 chromosomes). One of 302 completely plated marine sticklebacks collected from LITC was also heterozygous at both markers and gave an estimated allele frequency of 0.2% (1/604 chromosomes). These experiments confirm that the alleles shared by low-plated fish are also present at modest levels in completely plated migratory marine fish and suggest that worldwide evolution of the low-plated phenotype has occurred mainly by recurrent local selection for a family of alleles repeatedly brought into freshwater populations by marine founders.

Size and age of the shared genomic region in low-morph populations. To define the boundaries of the region of shared ancestry in different low-plated fish, we compared the sequence of *Eda* exons and selected flanking regions in different populations. Haplotype analysis showed that most low-plated populations shared characteristic sequences at positions extending from exon 1 of *Eda* through the flanking genes *Tumor necrosis factor (ligand) superfamily member 13b* (*Tnfsf13b*), *Glycoprotein A rich protein* (*Garp*), and *Gap junction protein beta 1* (*Gjb1*) (Table 1). However, low morphs from some populations resembled marine animals at either exon 1 of *Eda*, near the final exon of *Garp*, or at the sole exon of *Gjb1* (Table 1). These recombinant haplotypes show that the minimal interval conserved in low-plated fish is 16 kb, which spans intron 1 of *Eda* through the end of the coding region of *Garp*. Information from the entire conserved low haplotype is present in heterozygous form within the migratory marine individual from the Little Campbell River (LITC58) (Table 1).

To estimate the age of the shared haplotype, we examined the rate of neutral sequence evolution at third–base pair positions from all genes (*EDA* to *Garp*) in the shared region. The number of synonymous base pair substitutions per synonymous site (K_s) in the complete (SRMA) and low-morph (PAXB) haplotypes was larger than the number observed between Japan Marine (Pacific Ocean) (JAMA) and Japan Sea (JASE) [thought to have diverged ~2 million years ago on the

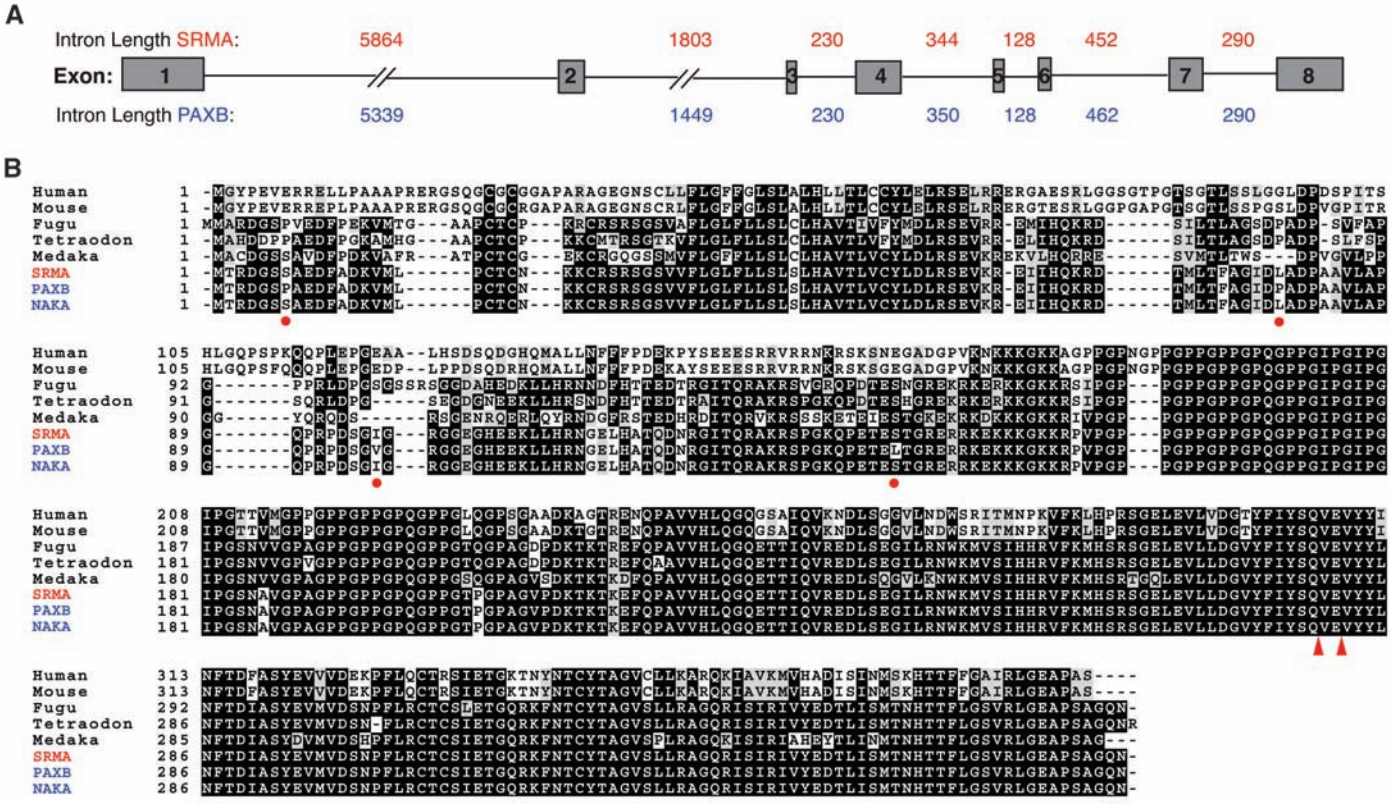

Fig. 2. Structure and sequence of stickleback Ectodysplasin. (**A**) The stickleback *Eda* locus is shown with exons shaded. Intron lengths differ in the marine (SRMA) and freshwater (PAXB) fish. (**B**) Alignment of EDA protein sequences from human, mouse, *Fugu*, *Tetraodon*, *Medaka*, SRMA (complete morph), PAXB (low morph), and NAKA (low morph) sticklebacks. Red arrowheads mark the two amino acids that differ in EDA-A1 and EDA-A2 splice forms. Red dots indicate amino acids different between PAXB and SRMA. Identical amino acids are shaded black, and conservative substitutions are shaded grey.

basis of genetic and geological estimates (*33*)], but smaller than the number between *G. aculeatus* (SRMA) and the sister species *G. wheatlandi* [thought to have diverged ~ten million years on the basis of the split between Pacific and Atlantic populations (*8*)] (K_s values of 0.01938, 0.01384, and 0.05875, respectively). The common clade of low-plated alleles thus probably arose between 2 and 10 million years ago. This date is at least 100 to 1000 times older than the recent divergence of modern stickleback populations in postglacial lakes and streams that formed at the end of the last Ice Age.

EDA-A1 transgenic sticklebacks develop extra lateral plates. Although the previously established role of EDA signaling in dermal bone and scale development (*23*) makes *Eda* a compelling candidate for the locus controlling lateral plate phenotypes, several other genes are present in the shared haplotype found in most low-plated populations. To test directly whether changing levels of EDA signaling could alter plate development in sticklebacks, we injected one-cell embryos from low-plated parents with a full-length mouse EDA-A1 cDNA under the control of the broadly expressed human cytomegalovirus (CMV) promoter. This construct has previously been shown to restore development of teeth, hair, and sweat glands when introduced into *Tabby* mutant mice carrying a null mutation at the *Eda* locus (*34*). Because of the mosaic inheritance of injected DNA constructs in transgenic sticklebacks (*35*), we scored injected and control siblings for mosaic patches of ectopic lateral plate formation after raising animals to 33- to 40-mm standard length. PCR genotyping confirmed that all fish from the cross were homozygous for an indel marker in intron 1 of *Eda* that is characteristic of the low-morph allele. Of 23 injected animals, 14 were also positive for the transgene (Fig. 4B). All 33 control siblings displayed the expected low-morph phenotype, having eight or fewer anterior plates per side (Fig. 4A). However, 3 out of 14 transgene-positive animals developed extra plates on their sides and caudal peduncles. The animal shown in Fig. 4, C and D, had six extra plates on its left side and one keel plate on its right peduncle. The other transgenic fish with additional plates also displayed them in a mosaic fashion, with one fish having one extra plate on the right side and two keel plates on the left peduncle and the other animal having one extra plate on the right side and two extra plates on the left side. No extra plates developed after injection of low-plated embryos with constructs containing the same vector backbone and a GFP insert instead of the EDA cDNA (*35*). These results confirm that EDA signaling is sufficient to trigger lateral plate formation on both the flank and tail regions of the body and that introduction of *Eda* transgenes can partially rescue the low-plated phenotype of freshwater sticklebacks.

Mutational spectrum in mammals and sticklebacks. In both humans and mice, a diverse set of mutations in three different genes of the EDA signaling pathway cause nearly indistinguishable defects in hair, teeth, sweat glands, and bone (*21*, *22*). In contrast, our studies show that widespread evolution of low-plated phenotypes in sticklebacks has occurred by repeated selection of a small number of related alleles at *Eda*. Several factors may account for this narrow spectrum of alleles that produce the low-plated phenotype. The diverse human *Eda* mutations (*25*, *26*), originally identified in rare patients with a medical disorder, have pleiotropic effects that would decrease fitness in the wild. By contrast, the low-plated phenotype in sticklebacks represents the first known example whereby specific *Eda* alleles have been repeatedly fixed by natural selection in the wild. The limited amino acid changes seen in most low-plated fish and the absence of any amino acid changes in the NAKA population suggest that natural variation in lateral plates is likely caused by coding or regulatory alleles of *Eda* that are not null mutations. Such limited changes may avoid the pleiotropic effects of a complete loss-of-function of *Eda* and thereby produce a spectrum of mutations very different from those found in human patients.

In addition, the repeated fixation of a particular haplotype at the *Eda* locus could be due to coselection for additional phenotypes produced by genes closely linked to *Eda*. Low-plated populations show correlated changes in salt tolerance (*36*), parasite susceptibility (*37*), and behavior (*38*, *39*). Although our transgenic results show that changes in lateral plate patterning are controlled by *Eda* itself, the multiple linked coding and noncoding changes in the common haplotype could also influence the structure or regulation of *Tnfsf13b*, *Garp*, or *Gjb1*. *Tnfsf13b* has previously been implicated in B cell development (*40*) and parasitic worm load in humans (*41*). The functions of *GARP* are unknown, but *Gjb1* belongs to a family of connexins that can produce changes in myelination and conduction velocity of neurons, hearing, skin thickness, and salt secretion (*42*). Related nonplate phenotypes caused by closely linked genes may contribute to the overall selective advantage of the ancient low-morph haplotype in freshwater (*36*).

Table 1. Distinctive haplotypes surrounding *Eda* in completely and low-plated sticklebacks. Genotypes are shown at 22 selected SNP positions throughout the plate morph region. Ten different completely plated (red) populations (AKMA, GJOG, JAMA, JASE, LITC, LLOY, NEU, NHR, SRMA, NAV) and four low-plated (blue) populations (BLAU, PAXB, WMSO, SFC) differ at all 22 positions, defining a shared haplotype characteristic of most complete and low populations. Recombinant haplotypes in SCX, FADA, PAXL, NOST, WALL, AKST, COND, SRST, and FRIL populations define a minimum shared haplotype, including SNPs 5-15. SNP genotypes in the completely plated LITC58 animal confirm that low-plated alleles are present in migratory marine populations. SNPs are either intergenic (I), synonymous (S), or nonsynonymous (N) changes and are located at the following nucleotide positions in the CHORI213-2D11 BAC sequence: 195422 (EDA Ser7Pro), 195496 (EDA Ser31Ser), 195649 (EDA Leu79Pro), 195692 (EDA Ile97Val), 203488 (EDA Ser140Leu), 203815 (EDA Pro172Pro), 207116 (TNFSF13B Leu69Phe), 208941 (TNFSF13B Gly197Gly), 208557 (TNFSF13B Ile219Ile), 210237 (GARP Met197Leu), 210240 (GARP Leu198Leu), 211414 (GARP Ser589Ile), 211415 (GARP Ser589Ile), 211573 (GARP Phe642Cys), 211615, 211677, 221853, 221944 (GJB1 His16His), 222209 (GJB1 Val105Ile), 222692 (GJB1 Thr266Ala), 222758, and 222759.

	EDA						TNFSF13B			GARP					Intergenic			GJB1			Intergenic	
Exon:	1	1	1	1	3	4	2	6	6	2	2	2	2	2	-	-	-	1	1	1	-	-
Type of Change:	N	S	N	N	N	S	N	S	S	N	S	N	S	N				S	N	N		
SNP:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
10 COMPLETE POPULATIONS	T	T	T	A	C	G	G	C	C	A	T	G	C	T	T	C	T	C	G	A	G	T
4 LOW POPULATIONS	C	C	C	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	G	A	G
SCX (26)	T	T	T	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	G	A	G
FADA (23)	T	T	T	A	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	G	A	G
PAXL (8)	T	T	T	A	T	A	C	T	T	C	C	T	T	G	A	G	A	T	G	A	G	T
NOST (24)	C	C	C	G	T	A	C	T	T	C	C	T	T	G	A	C	T	C	G	A	G	T
WALL (4), AKST (5), & COND (12)	C	C	C	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	G	A	G	T
SRST (9)	C	C	C	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	A/G	G/A	T/G
FRIL (15)	C	C	C	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	G	G/A	T/G
OMPL(19)	C	C	T	G	T	A	C	T	T	C	C	T	T	G	A	G	A	T	A	G	A	G
LITC58 (10)	T/C	T/C	T/C	A/G	C/T	G/A	G/C	C/T	C/T	A/C	T/C	G/T	C/T	T/G	T/A	C/G	T/A	C/T	G/A	A/G	G/A	T/G
NAKA (1)	T	T	T	A	C	G	G	C	C	A	T	G	C	T	T	C	T	C	G	A	G	T

Mechanism for rapid parallel evolution. Forty years ago, Lindsey proposed that parallel evolution of stickleback lateral plate patterns could occur either by independent mutations in different populations or by repeated selection on the standing genetic variation already present in marine ancestors (*43*). Our studies show that both mechanisms have contributed. The EDA sequences in low-plated NAKA fish are distinct from those in other low populations (Fig. 3B). However, the presence of a shared haplotype in most low-plated populations suggests that selection on standing variation is the predominant mechanism underlying the recent rapid evolution of changes in lateral plate patterns in wild sticklebacks.

One surprising finding of this study is the enormous geographical distance over which related low-plated alleles are shared. Both nuclear and mitochondrial data (*9*, *31*, *32*) rule out the possibility that these alleles have spread by migration of a single low-plated population of fish throughout the world. In contrast, our surveys of large numbers of marine fish show that low-plated alleles are present at detectable frequencies in completely plated sticklebacks. This low frequency is likely maintained by the occasional hybridization that occurs when marine sticklebacks come into contact with low-plated freshwater populations in coastal streams during the breeding season (*38*) or perhaps by fitness effects in heterozygotes. The annual migration of marine sticklebacks from ocean to freshwater could provide a mechanism for repeated flow of the ancient low-morph alleles back into marine populations and, from there, into new freshwater environments. Fish heterozygous for completely and low-plated alleles at *Eda* typically have either complete or partial lateral plates (*4*, *6*), facilitating the persistence of a cryptic genetic variant within marine populations, and providing a genetic mechanism that could lead to rapid emergence of the low-plated phenotype after colonization of new environments.

The differences in armor plate patterns of sticklebacks are so large that Cuvier originally classified plate morphs as distinct species (*10*).

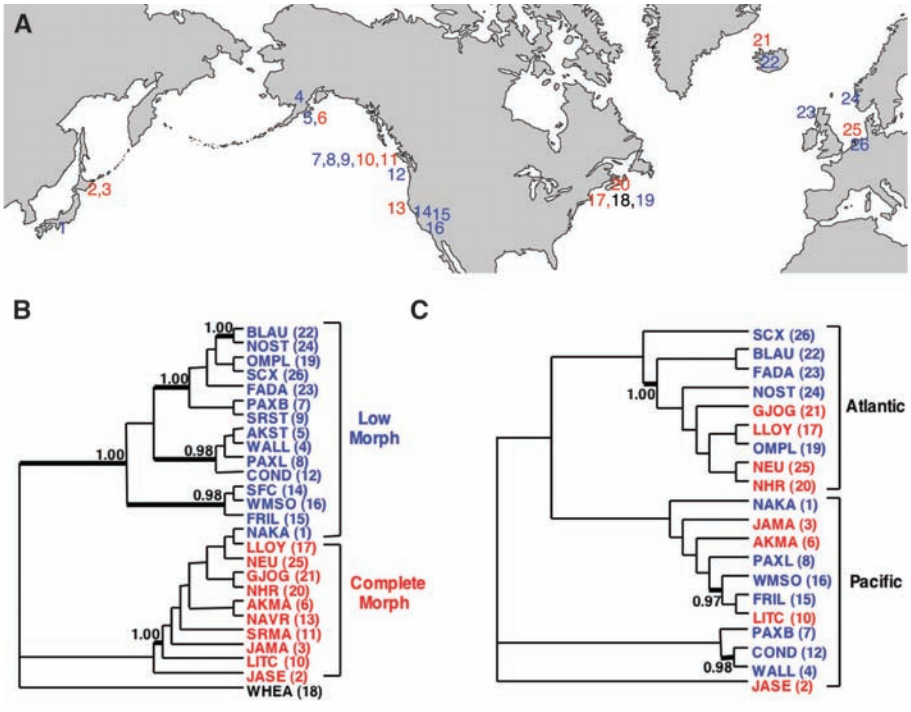

Fig. 3. Most low-plated populations have a shared history at the *Eda* locus, but not at other nuclear genes. (**A**) Geographic origin of 25 low-plated (blue numbers) and completely plated (red numbers) stickleback populations used for sequencing. (**B**) MrBayes tree of *Eda* sequences. *G. wheatlandi*, a sister species of *G. aculeatus*, was used as an outgroup (black, number 18). *Eda* alleles in most low populations share a common origin. Posterior probabilities greater than 95% are shown (branches in bold). (**C**) MrBayes phylogeny based on genetic differences in 193 SNPs from 25 random nuclear loci. Tree topology is significantly different from the EDA tree, ruling out the possibility that all present-day low-plated populations are derived from a single population sharing alleles at most genes.

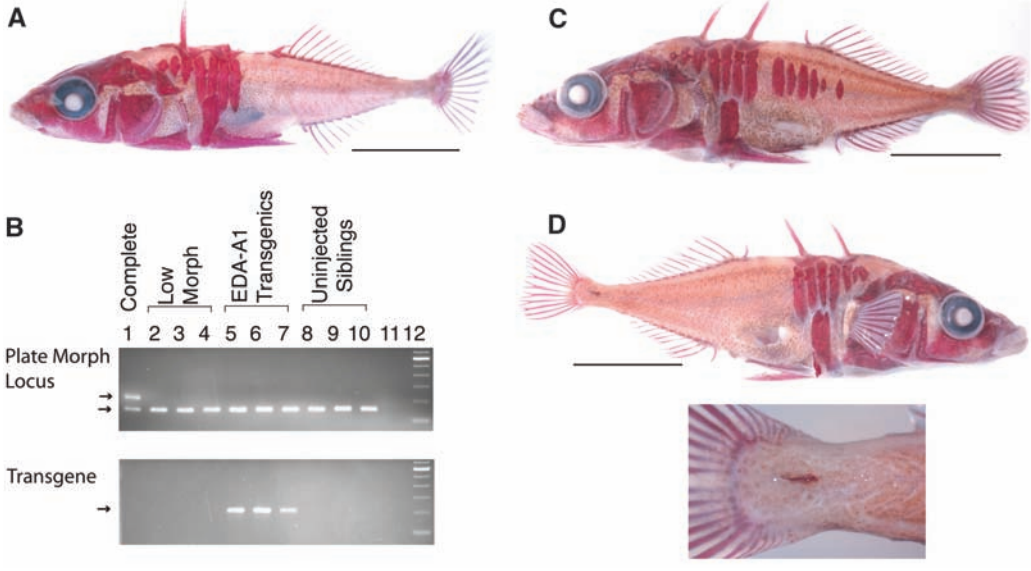

Fig. 4. EDA-A1 stimulates lateral plate formation in transgenic sticklebacks. (**A**) Typical low-plated pattern in control fish stained with alizarin red. (**C** and **D**) A sibling from the same clutch after introduction of a transgene containing a full-length mouse EDA-A1 cDNA driven by a CMV promoter (*34*). Note six extra lateral plates that have formed in the flank region on the left side (C), and the new keel plate on the right tail (D). Differences in spine number are due to clipping for DNA recovery. Scale bars, 1 cm. (**B**) (top) PCR genotyping confirms that transgenic animals are homozygous for a 150-bp allele characteristic of the ancestral low-plated allele at the endogenous *Eda* locus, just like wild-caught low-plated fish from FRI, San Francisquito Creek (SFC) and uninjected siblings. In contrast, completely plated fish from FRI are either heterozygous for the 218-bp allele and the 150-bp allele (lane 1) or homozygous for the 218-bp allele (not shown). (B) (bottom) Primers specific for mouse EDA-A1 amplify a 211-bp fragment from transgenic fish, but not from uninjected control animals. Genomic DNA samples: Lane 1, FRI Complete; 2, FRI Low; lanes 3 to 4, SFC Low; lanes 5to 7, EDA-A1 transgenic animals with additional lateral plates; lanes 8 to 10, uninjected siblings of EDA-A1 transgenic animals; lane 11, water control; lane 12, 100-bp DNA size ladder (Invitrogen).

In the past, it has been difficult to trace such large-scale changes in body form to the action of particular genes and mutations. Our results show that large changes in vertebrate skeletal morphology in the wild can be created by relatively simple genetic mechanisms. Natural populations have variants of the same major signaling genes already known to play a crucial role in normal development and human disease. The variant alleles preexist at low frequency in ancestral marine populations and can be repeatedly fixed in new environments to produce a rapid shift to an alternative body form. Many other dramatic morphological, physiological, and behavioral changes are known in sticklebacks. With the recent advent of forward genetic approaches in this system, it should now be possible to identify the genes that underlie many ecologically important traits, which will bring a new molecular dimension to the study of how phenotypic changes arise during vertebrate evolution.

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Supporting Online Material

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Temporal Relationships of Carbon Cycling and Ocean Circulation at Glacial Boundaries

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Evidence from high-sedimentation-rate South Atlantic deep-sea cores indicates that global and Southern Ocean carbon budget shifts preceded thermohaline circulation changes during the last ice age initiation and termination and that these were preceded by ice-sheet growth and retreat, respectively. No consistent lead-lag relationships are observed during abrupt millennial warming events during the last ice age, allowing for the possibility that ocean circulation triggered some millenial climate changes. At the major glacial-interglacial transitions, the global carbon budget and thermohaline ocean circulation responded sequentially to the climate changes that forced the growth and decline of continental ice sheets.

Records of past global climate preserve evidence of large-scale changes in temperature and ice volume at glacial-interglacial boundaries. Although the timing of ice ages is broadly driven by Milankovich orbital cycles, the small insolation changes require amplifying mechanisms to produce the large glacial-interglacial climate changes. Fluctuation in North Atlantic Deep Water (NADW) production is a potential amplifier and has been suggested as a trigger for rapid global climate shifts (*1*). Carbon dioxide (CO_2) is another possible amplifier on glacial-interglacial time scales, because its atmospheric concentration is predominantly controlled by changes in deep-ocean storage (*2*) and the terrestrial carbon reservoir (e.g., *3*–*5*). Carbon isotope ratios are distinct in different reservoirs, making it a powerful tool to constrain the timing of global carbon budget reorganizations relative to other changes in the climate system. In the oceans, carbon isotope ratios of benthic foraminifera (benthic $\delta^{13}C$) are commonly used as a proxy for ocean circulation because they vary systematically in water masses [e.g., (*6*–*8*)]. However, temporal benthic $\delta^{13}C$ changes at any location reflect a combination of the global carbon mass balance, ocean-circulation changes, air-sea equilibration, and productivity changes. If carbon budget and ocean-circulation signals can be deconvolved, the temporal sequence of major shifts in global ice volume, carbon mass balance, and ocean circulation can help to clarify the ocean's role as a trigger of, or a response to, major climate changes. In this study, we compare the temporal sequence of these changes since the last interglacial period. Chronological ambiguities are obviated through study of different proxy signals in the same core, and thus the sequence of events associated with climate change and ocean circulation can be extracted.

Nd isotopic systematics. We use Nd isotopes as a proxy of the balance between NADW and southern-sourced waters in the South Atlantic. $^{143}Nd/^{144}Nd$ ratios vary in the

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