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Report

A Genome-wide SNP Genotyping Array Reveals Patterns of Global and Repeated Species-Pair Divergence in Sticklebacks

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Summary

Genes underlying repeated adaptive evolution in natural populations are still largely unknown. Stickleback fish (*Gasterosteus aculeatus*) have undergone a recent dramatic evolutionary radiation, generating numerous examples of marine-freshwater species pairs and a small number of benthic-limnetic species pairs found within single lakes [1]. We have developed a new genome-wide SNP genotyping array to study patterns of genetic variation in sticklebacks over a wide geographic range, and to scan the genome for regions that contribute to repeated evolution of marine-freshwater or benthic-limnetic species pairs. Surveying 34 global populations with 1,159 informative markers revealed substantial genetic variation, with predominant patterns reflecting demographic history and geographic structure. After correcting for geographic structure and filtering for neutral markers, we detected large repeated shifts in allele frequency at some loci, identifying both known and novel loci likely contributing to marine-freshwater and benthic-limnetic divergence. Several novel loci fall close to genes implicated in epithelial barrier or immune functions, which have likely changed as sticklebacks adapt to contrasting environments. Specific alleles differentiating sympatric benthic-limnetic species pairs are shared in nearby solitary populations, suggesting an allopatric origin for adaptive variants and selection pressures unrelated to sympatry in the initial formation of these classic vertebrate species pairs.

Results and Discussion

Many traits in plants and animals evolve repeatedly in response to similar environmental conditions, suggesting that they are adaptive and shaped by natural selection [2]. Marine three-spine sticklebacks colonized and adapted to a large number of new freshwater environments at the end of the last ice age, providing an outstanding opportunity for identifying the genetic and genomic basis of repeated evolutionary change in natural populations. A variety of molecular tools have recently been developed for sticklebacks, including microsatellite-based linkage maps, physical maps, restriction site-associated DNA polymorphisms, and a whole-genome sequence assembly [3–5]. To facilitate the further genetic and genomic characterization of stickleback diversity, we designed a genotyping array with 3,072 well-spaced SNPs identified from expressed sequence tags (ESTs) and genomic sequences from different populations (see Supplemental Experimental Procedures available online). We used this platform to study patterns of variation found in 196 individuals from a worldwide collection of 34 different stickleback populations, bracketing both the large geographic range and extreme morphological diversity of the species complex ([1, 6, 7]; Figure 1A; Table S1). A total of 1,159 SNPs gave good genotyping signals and were variable among the populations surveyed (Figures S1A and S1B; Table S2). Based on the overall level of polymorphism observed within and among global populations (Figures 1A, 1B, and S1B), the arrays should provide a useful platform both for mapping phenotypic traits in laboratory crosses ([8, 9]; Supplemental Experimental Procedures) and for studying relationships between populations using a genome-wide set of markers.

Patterns of Variation within and among Global Stickleback Populations

Our survey of 34 populations shows that mean heterozygosity is significantly higher in marine than in freshwater populations (0.200 versus 0.139; $p = 0.017$), with some freshwater populations showing very low diversity (Figure 1B). Lower heterozygosity in freshwater populations may reflect smaller effective population sizes and varying demographic histories involving bottlenecks during the recent colonization of freshwater habitats (see also [10–12]) but was not significantly associated with lake size, elevation, or distance from the sea in the current study ($p > 0.05$ all tests).

High phenotypic diversity among sticklebacks is mirrored by a relatively high degree of genetic differentiation among populations (mean pairwise $F_{ST} = 0.193$; range 0.031–0.383; Figure S1C). Principal component analysis (PCA) revealed a geographic split between Atlantic and Pacific basins as a major axis of variation in genome-wide SNPs (18 percent variation explained [PVE]; Figure 1C). This is consistent with a deep split between Atlantic and Pacific lineages observed with a smaller set of nuclear markers [13]. PC2 describes variation among Pacific populations and divides northern freshwater from marine and southern freshwater populations (Figure 1C).

Both neutral and selective processes can lead to phenotypic and genomic differentiation. To identify signatures of adaptive genomic differences, we looked for loci consistently differentiated in stickleback species pairs that have evolved repeatedly in multiple locations, including marine-freshwater pairs found in many different marine and lake/stream environments and benthic-limnetic pairs found in a small number of isolated lakes in British Columbia.

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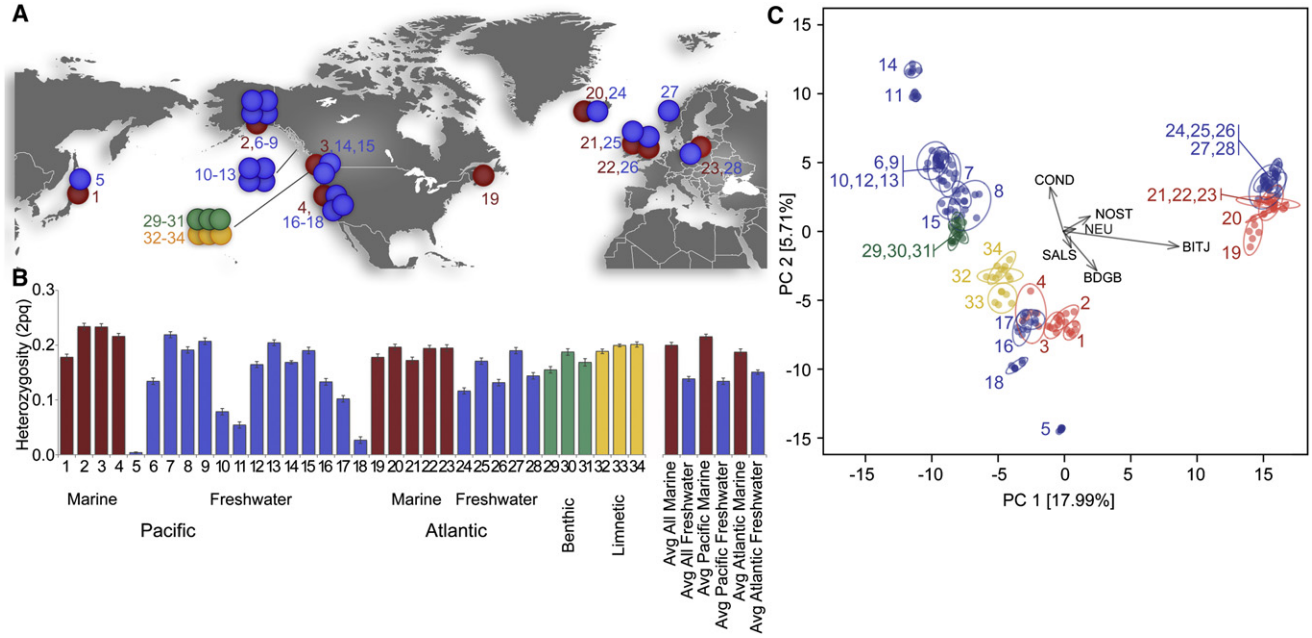

Figure 1. Levels of Heterozygosity and Patterns of Genetic Variation among Global Stickleback Populations

(A) Marine (red) and freshwater (blue) sticklebacks were sampled across the Northern Hemisphere. Benthic (green) and limnetic (yellow) species pairs are restricted to a few lakes in British Columbia. See Table S1 for population information.

(B) Mean heterozygosity levels (2pq ± SEM) within freshwater populations are significantly lower than within marine populations.

(C) Principal component analysis of global populations reveals the first major axis of variation separating Pacific (left cluster) from Atlantic (right cluster) populations, and a second axis separating Northern Pacific freshwater populations from Southern Pacific freshwater and Pacific marine populations. SNP ascertainment vectors are shown as gray arrows (see Supplemental Experimental Procedures). See also Figure S1 and Tables S1 and S2.

Genome Scans in Marine-Freshwater Species Pairs

Marine and freshwater sticklebacks represent genetically, morphologically, and behaviorally distinct ecotypes that have evolved repeatedly throughout the Northern Hemisphere [7, 14]. To identify potential adaptive loci that underlie repeated ecological differentiation, we searched for SNPs that consistently distinguish marine and freshwater ecotypes, using the method of Coop et al. [15] to correct for overall structure between populations (Figure 2; Table S3). Five of the six top-scoring SNPs fell near or within genomic regions previously associated with marine and freshwater ecotypes [4, 13, 16, 17], including two SNPs (chrIV:12,811,933 and chrIV:12,815,024; candidate class) near the ectodysplasin (*EDA*) region underlying variation in armor plate phenotypes, and three SNPs (chrI:21,663,978, chrI:21,689,292, and chrI:21,487,034; candidate class) near the *Na/K ATPase* (*ATP1a1*) gene for salinity tolerance [18] that shows strong differentiation along a marine-freshwater salinity gradient in Scotland [16]. Although these genes have already been linked to morphological or physiological differences between particular marine and freshwater populations, allele frequencies have not previously been characterized in multiple individuals from a global set of populations. The repeated differentiation of these regions in many marine and freshwater sticklebacks provides strong additional support for the adaptive significance of these loci even after correcting for potential nonindependence of populations—a confounding factor not explicitly adjusted for in previous studies [4, 13].

Our analysis also identified a new marker associated with marine-freshwater differentiation on chromosome 2. This outlier SNP (chrII:418,094; candidate class) is located near multiple, potentially duplicated genes belonging to the mucin gene family (Table S3; [19, 20]). Mucus secretions in fish play important roles in osmoregulation, locomotion, and protection against pathogens [21]. Genetic differences in epithelial barrier functions seem likely in marine and freshwater fish, and we propose that variation in the mucin cluster contributes to repeated transformation of marine sticklebacks to freshwater forms living in low-ionic-strength environments.

To determine whether we detect similar or different adaptive loci in Pacific and Atlantic sticklebacks, we also performed analyses separately in fish from each ocean basin. SNPs in the *EDA* and *ATPase* region showed high Bayes factor scores (BF > 3.0) in sticklebacks from both basins (Figure 2B). The mucin gene region SNP scored even higher than *EDA* and *ATPase* loci in Atlantic sticklebacks but was only weakly differentiated in Pacific sticklebacks. In contrast, four SNPs located on chromosomes IV, IX, XI, and XVII had BF > 2.5 in Pacific, but not Atlantic, populations. These SNPs are located near genes that influence iron metabolism in humans and fish (chrIV:12,022,250; general class; Figure 2F; *ABCB7* gene [22, 23]); alter blood pressure and protect the heart, vasculature, and lungs from oxidative stress (chrIX:8,719,760; candidate class; Figure 2I; *SOD3* gene [24–26]); control ion gradients involved in sperm motility, resorption of bone, and digestion of microbes by phagocytes (chrXI:5,708,414; candidate class; Figure 2J; *ATP6V0A1* gene [27, 28]); and influence immune functions, pathogen clearing, and expression levels of a cell surface mucin gene required for epithelial barrier function and protection against infections (chrXVII:9,697,366; general class; Figure 2K; *PRKCD* gene [29–33]). Given the low density of the genotyping array, the actual targets of selection might be other linked genes (see Table S3). However, we find it interesting that SNPs linked to mucin functions were recovered in both basins, highlighting the likely importance of epithelial barrier

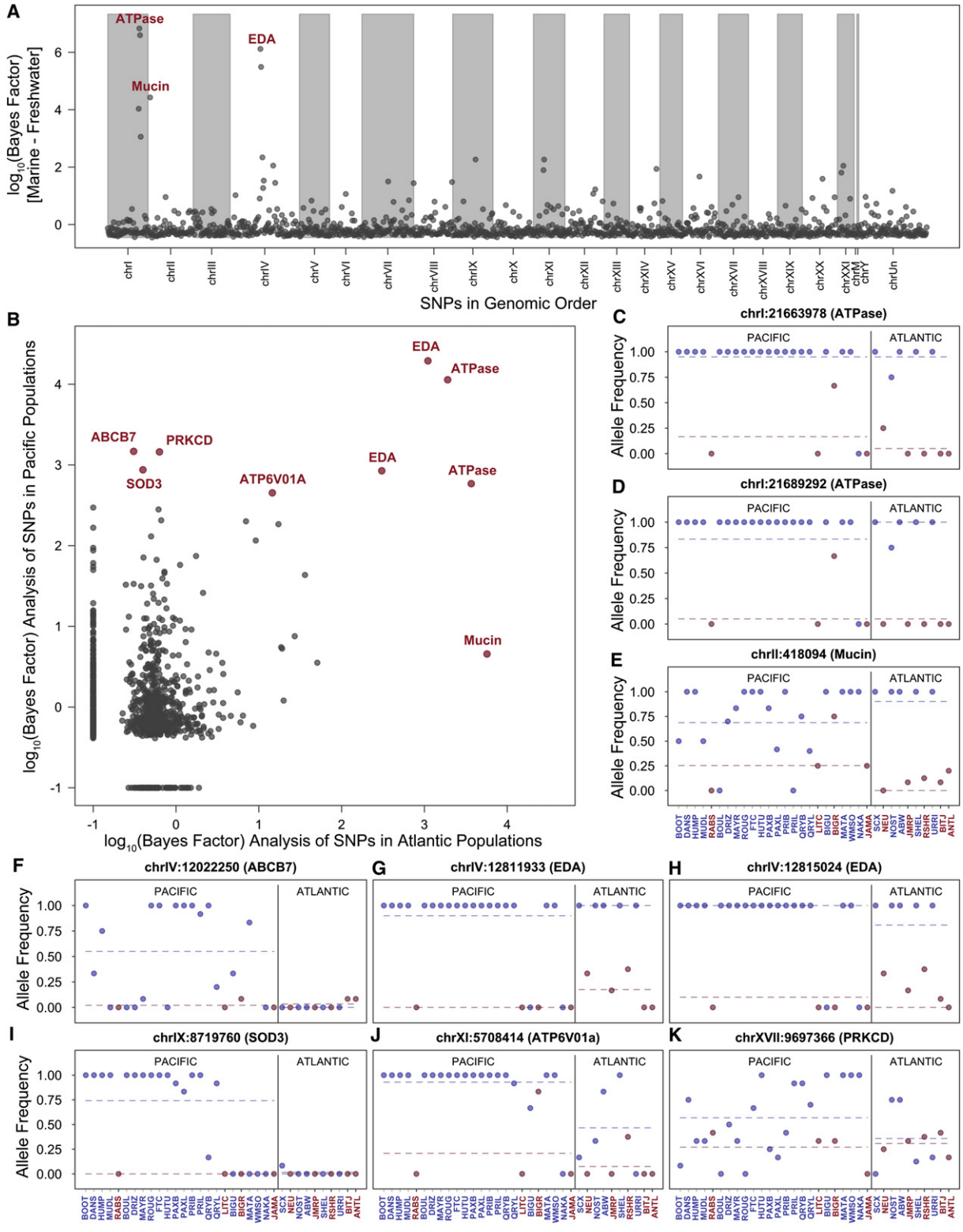

Figure 2. Bayesian Scan for Genomic Regions Consistently Differentiated between Marine and Freshwater Environments

(A) Bayes factor scores for all markers in the genome, with the top-scoring regions (*ATPase*, *EDA*, and mucin gene cluster regions on chromosomes I, IV, and II, respectively) indicated in red. (All SNPs with $\log_{10}$ Bayes factors $\geq$ 1.5 are listed in Table S3.)

(B) Bayes factors scores found when Pacific and Atlantic sticklebacks are analyzed as separate regional groups. SNPs with Bayes factors > 2.5 in either population are labeled with corresponding gene region in red.

(C–K) Allele frequencies in each of the 34 global populations for top-ranking SNPs highlighted in (A) and (B) (freshwater populations, blue points; marine populations, red points; mean freshwater and marine allele frequency, blue and red dashed lines, respectively; Pacific and Atlantic populations are grouped on the left and right side of the vertical solid gray line).

changes during recurrent evolution of marine and freshwater sticklebacks.

Recurrent genetic differentiation suggests that marine-freshwater stickleback adaptation proceeds in part by large shifts in allele frequency at some loci shared across multiple populations (Figure 2). Many additional adaptive variants may exist that are specific to individual populations, and these would not be detected by the current method. Given the average spacing between markers in our study (approximately one marker per 400 kb), we may have missed other strongly selected loci in the genome, even if they are repeatedly used in different populations. Previous studies have shown that linkage disequilibrium extends approximately 20–40 kb around major genes controlling armor and pelvic traits in natural populations [11, 13]. Selective sweeps that are tightly linked to the genotyped SNPs and sweep events that are young, strong, or fall within regions of suppressed recombination are therefore the most likely to be detected in genome scans for adaptive loci using this SNP genotyping platform. Our results do not preclude the possibility that marine-freshwater adaptation might also involve other loci with smaller shifts in allele frequency (we note that a number of other markers show high or moderately high Bayes factors; Figure 2B; Table S3). Nevertheless, these findings highlight how repeated evolution can be used to filter genetic drift from the signatures of adaptive loci and show the potential of this approach for future studies using even higher densities of markers or whole-genome sequencing.

Genome Scans in Benthic-Limnetic Species Pairs

A second example of species pairs in sticklebacks is much less common than marine-freshwater pairs but provides one of the best-studied examples of ecological speciation in vertebrates. In a few lakes in British Columbia, sticklebacks occur as distinct open-water (limnetic) and bottom-dwelling (benthic) forms [14, 34–36]. These sympatric forms are distinguishable by heritable differences in size, shape, feeding morphology, body armor, mate preference, and behavior, which confer fitness advantages when tested in the corresponding open-water and near-shore environments [37, 38]. Although fish with hybrid morphology can be found at low frequencies in the species-pair lakes, hybrids appear less fit than parental forms in both environments [37, 39]. Patterns of variation in mitochondrial markers and a small number of autosomal microsatellite markers suggest that these species pairs have evolved multiple times in different lakes, rather than originating once and spreading to multiple lakes (although the data are also inconsistent with benthic-limnetic monophyly within each lake [12, 40, 41]). We reinvestigated this classic example of recurrent ecological speciation on a genome-wide scale, scanning for loci that remain differentiated between benthic and limnetic forms despite ongoing homogenization of neutral regions of the genome due to hybridization in sympatry [41].

We used F_{ST} outlier tests [42, 43] to identify loci with higher or lower levels of differentiation between limnetic and benthic pairs than expected under a neutral model. Separate tests were performed on each of three lakes and identified 46 SNPs as outliers linked to putatively selected regions (false discovery rate $\leq$ 0.05; Table S4). These comprise 2.5%, 4.3%, and 1.9% of polymorphic SNPs surveyed in Paxton, Priest, and Little Quarry (hereafter "Quarry") lakes, respectively, indicating that multiple loci (but a small proportion overall) are involved in benthic-limnetic species-pair divergence via large shifts in allele frequency. Fifteen SNPs were outliers in two or more lakes (12 general class, 3 candidate class), four of which were outliers in all three lakes (3 general class, 1 candidate class) (Figure 3; Table S4). For comparison, only 1.4 and 0.01 SNPs would be expected to be parallel selected by chance in two and three lakes, respectively. As parallel outliers across lakes, these SNPs tag regions that are common and therefore likely important to the repeated evolution of benthic-limnetic species pairs (see Table S5 for a summary of nearby genes).

One outlier SNP (chrXIX:10,552,047; candidate class) is linked to *KITLG*, a gene previously shown to control lighter gill and skin pigmentation in Paxton benthic fish [44]. We find that *KITLG* is strongly differentiated in all three species-pair lakes, suggesting that genetic variation at this locus controls an adaptive trait repeatedly selected during formation of all benthic-limnetic species pairs (perhaps color matching to different light and dark water backgrounds typical of near-shore and open-water environments [45]).

Two other outlier SNPs (chrX:14,456,479 and chrX:14,549,101; general class) showing benthic-limnetic differences in all three lakes fall within a region that contains genes with roles in immune function and vision and flanked by large repeated clusters of >24 variable and constant domains of immunoglobulin light-chain genes (*IGK*; Figure S2). Benthic and limnetic ecotypes are known to differ in parasite loads [46]. Differential use of microhabitats and food sources likely leads to differential exposure to a variety of pathogens and strong selection on host defenses. Our results suggest that differences in immunity play a significant role in the evolution of benthic-limnetic species pairs and identify a specific genomic region likely contributing to immunological differences between species.

Evolution of Benthic-Limnetic Species Pairs

Factors influencing the origin and restricted distribution of benthic-limnetic species pairs in three-spine sticklebacks are not well understood. Previous studies have hypothesized that the limited distribution of benthic-limnetic species pairs could be due to rare "double invasion" events facilitated by fluctuations in sea level in British Columbia [12, 47]. The genome-wide data showed two features consistent with separate invasion events: lower average heterozygosity within the postulated older (benthic) members of the species pairs (Figure 1B; mean benthic and limnetic heterozygosity 0.170 versus 0.196; $p = 0.030$), and closer clustering of the postulated younger (limnetic) members of the species pairs with marine fish in the global PCA (Figure 1C). However, these differences could also arise from differences in effective population size of benthic and limnetic ecotypes, and recent geological data [48] suggest that the magnitude of local sea-level changes may not have been sufficient to mediate double invasions.

To further investigate species-pair relationships, we performed a PCA restricted to benthic and limnetic populations (Figure 4A). The first PC (15.15% PVE) clearly separated benthic and limnetic fish by different lakes of origin, consistent with the independent evolution of pairs in each of the three lakes. However, PC2, accounting for nearly as much variation (13.41%), separated all three benthic from limnetic types, suggesting similar genetic variants underlying benthic and limnetic differences in all three lakes.

When adaptive divergence occurs in the face of gene flow, the evolutionary history inferred from patterns of neutral variation can be very different from the evolutionary history inferred from variation at adaptive loci [13, 49]. We therefore divided

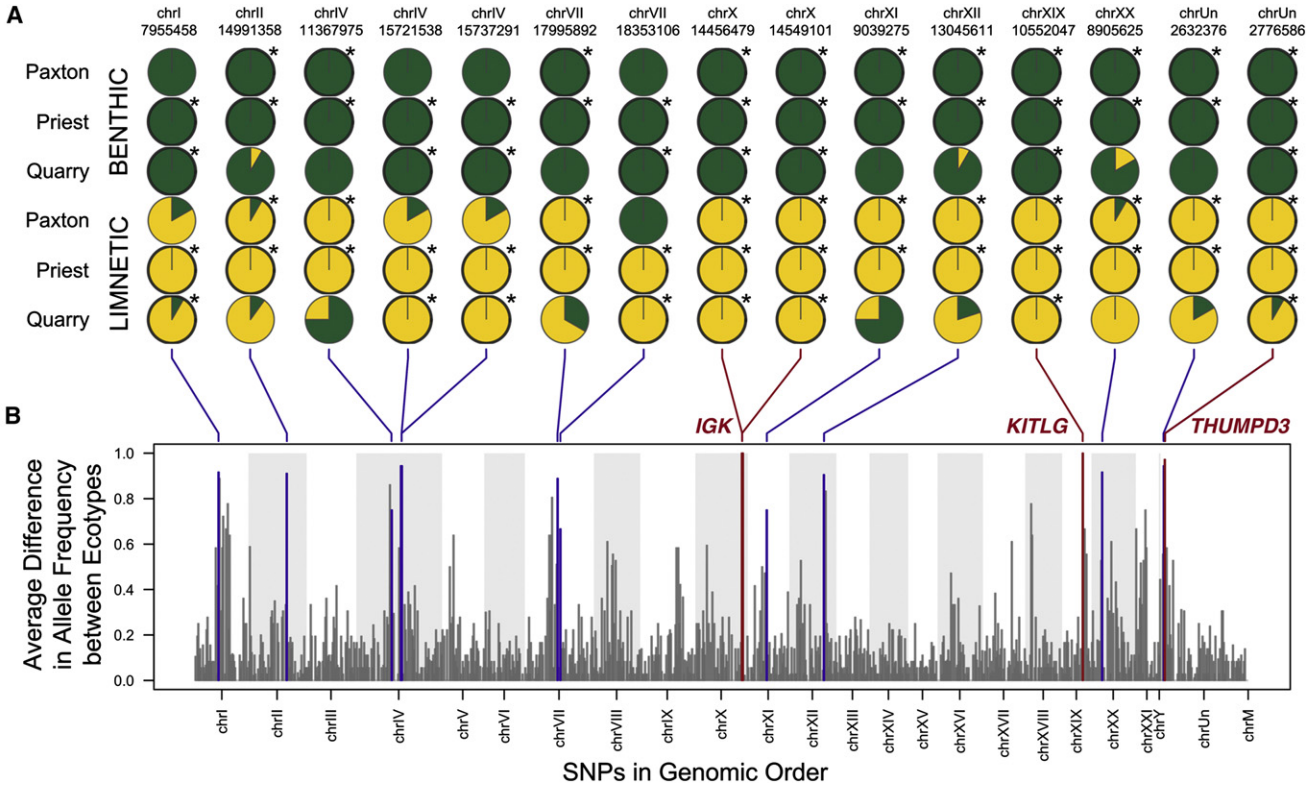

Figure 3. F_{ST} Outlier Analyses in Benthic-Limnetic Populations

(A) Allele frequencies of F_{ST} outlier SNPs are shown in pie charts for three species-pair lakes. Asterisks and dark outlines around pies indicate lakes in which SNP was found to be an F_{ST} outlier. Four SNPs appeared to be under selection in all three lakes (red lines; corresponding gene regions are indicated), and a further 11 SNPs appeared to be under selection in two of three lakes (blue lines).

(B) Genome graph of allele frequency differences between pooled ecotypes indicating chromosomal position of F_{ST} outliers. See Table S4 and Figure S2 for more details.

SNPs into a set of putatively selected (46 adaptive loci identified in any of the three different lake genome scans described above) and neutral markers (879 polymorphic SNPs among benthic and limnetic individuals from the three lakes). PCA of putatively neutral SNPs revealed clustering by lake rather than ecotype (Figure 4B). In contrast, PC1 of "selected" SNPs separated benthic and limnetic fish into ecotypic clusters (65% PVE; Figure 4C), highlighting the importance of a shared origin to adaptive genetic variation in these species pairs. Interestingly, when global populations were projected onto adaptive benthic-limnetic PC space, we observed a number of freshwater and marine populations that clearly overlapped with the divergent benthic (e.g., Pop15/FTC) and limnetic (e.g., Pop3/LITC) clusters, respectively (Figure 4C; see Table S1 for population details). In contrast, none of the global populations showed affinity with the clusters of genetic variation observed in PCA of neutral markers (Figure 4B).

Clustering of benthic and limnetic fish by ecotype suggests that some of the adaptive genetic variation in each lake arises from shared adaptive variants, rather than from novel mutations that have occurred separately in each lake. The allopatric single-ecotype populations that cluster with benthic and limnetic fish identify new examples of solitary populations that also carry similar allele assemblages. Interestingly, these solitary populations are located in geographic proximity to the lakes with species pairs. Such populations could represent allopatric source populations from which adaptive benthic and limnetic alleles originally arose and spread, or single-species populations in which ecological selection has also occurred for benthic-like or limnetic-like phenotypes, resulting in similar fixation of shared genetic variants that contribute to particular traits. Regardless, our results indicate that selection pressures unrelated to sympatry can also generate a substantial part of characteristic benthic or limnetic allele assemblies and suggest a more important role of allopatric adaptive divergence and reuse of standing genetic variation than previously recognized for the repeated evolution of the classic benthic-limnetic species pairs.

Conclusion

Studies in many organisms have demonstrated the benefits of being able to type multiple individuals at many markers in a fast and affordable way. The development of an informative genome-wide SNP array for sticklebacks will facilitate additional genetic mapping studies in this emerging model organism and assist studies of natural population diversity. Our genome scans for adaptive loci using the genome-wide array show that repeated formation of species pairs occurs in part by recurrent strong shifts in allele frequency at particular loci. These data confirm that strong differentiation has repeatedly occurred at some previously known loci (*EDA*, *ATP1a1*, *KITLG*). In addition, we identify many new regions that may contribute to repeated differentiation of either marine-freshwater ecotypes (e.g., markers linked to *ABCB7*, *ATP6V0A1*, mucin gene cluster) or benthic-limnetic ecotypes (e.g., markers near the immunoglobulin light chain gene cluster

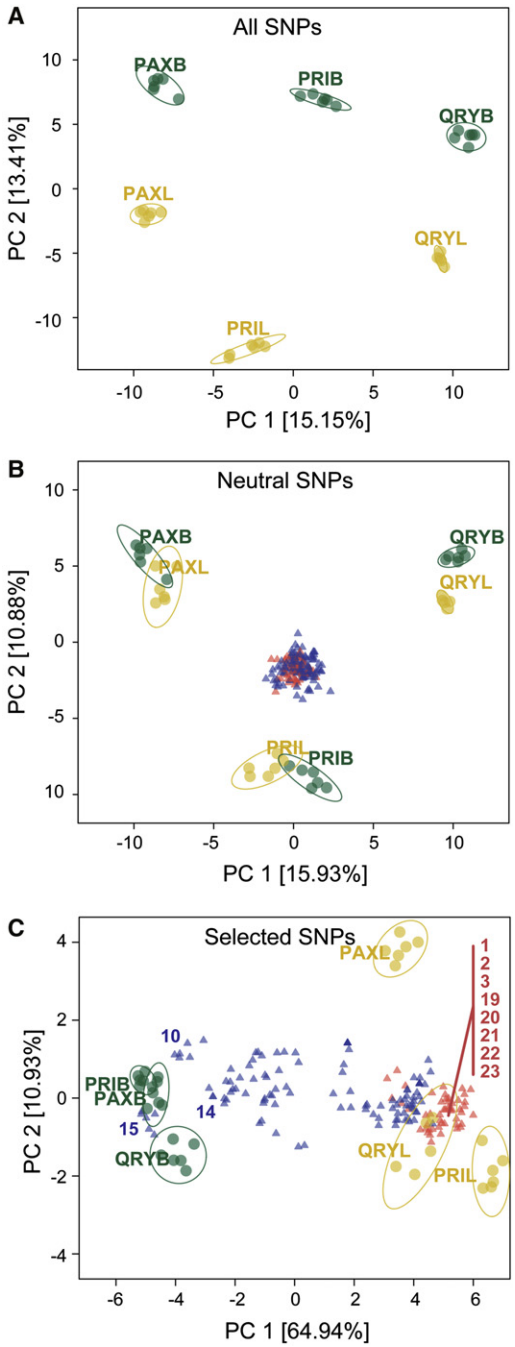

Figure 4. Principal Component Analyses of SNPs in Benthic-Limnetic Species Pairs and Global Populations

Positions of benthic (green) and limnetic (yellow) individuals are shown using all 925 polymorphic SNPs (A), 879 putatively neutral SNPs (B), or 46 putatively selected SNPs (C). Ellipses represent 95% confidence intervals for each ecotype within a lake. Global populations projected onto benthic-limnetic variation space in (B) and (C) are shown as red (marine) and blue (freshwater) triangles, with the benthic-like or limnetic-like populations labeled with a number code. See Table S1 for population information, Table S4 for information on SNPs linked to adaptive loci in each of the three lakes, and Figure S3 for the global distribution of alleles at SNPs linked to putatively adaptive loci.

[*IGK*] that are differentiated in all three species-pair lakes), which can now be further investigated to better understand the basis of selection and ecological adaptation. Finally, our studies illustrate how genotyping of a large number of loci in many different populations can identify novel relationships among the gene assemblages that have evolved in different geographic locations. Many of the outlier loci identified in benthic-limnetic genome scans not only are shared in two or more lakes but also are found in similar assemblages in nearby allopatric solitary populations. The markers identified in these studies provide a new foundation for studying the molecular basis of specific benthic-limnetic phenotypes, as well as the larger historic and geographic factors that have led to this classic example of recurrent ecological speciation in vertebrates.

Experimental Procedures

SNPs were ascertained primarily from large EST collections of marine and freshwater populations. Potential SNPs were genotyped on 196 individuals using an Illumina GoldenGate genotyping array platform. Analyses of allele frequency, heterozygosity, and F_{ST} [50] were performed using Perl scripts. PCA was performed in R (v2.11.1). Genome scans for SNPs with allelic correlation to marine-freshwater environments were performed using BayEnv [15], which allowed for correction for the potential nonindependence among populations. Genome scans for F_{ST} outliers among benthic-limnetic species pairs were performed using BayeScan 2.01 [42, 43]. See Supplemental Experimental Procedures for more details.

Accession Numbers

SNPs reported herein have been deposited in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) with the accession numbers specified in Table S2.

Supplemental Information

Supplemental Information includes five tables, three figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.11.045.

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