Symposium

Whole-Genome Sequencing Reveals That Regulatory and Low Pleiotropy Variants Underlie Local Adaptation to Environmental Variability in Purple Sea Urchins*

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abstract: Organisms experience environments that vary across both space and time. Such environmental heterogeneity shapes standing genetic variation and may influence species' capacity to adapt to rapid environmental change. However, we know little about the kind of genetic variation that is involved in local adaptation to environmental variability. To address this gap, we sequenced the whole genomes of 140 purple sea urchins (*Strongylocentrotus purpuratus*) from seven populations that vary in their degree of pH variability. Despite no evidence of global population structure, we found a suite of single-nucleotide polymorphisms (SNPs) tightly correlated with local pH variability (outlier SNPs), which were overrepresented in regions putatively involved in gene regulation (long noncoding RNA and enhancers), supporting the idea that variation in regulatory regions is important for local adaptation to variability. In addition, outliers in genes were found to be (i) enriched for biomineralization and ion homeostasis functions related to low pH response, (ii) less central to the protein-protein interaction network, and (iii) underrepresented among genes highly expressed during early development. Taken together, these results suggest that loci that underlie local adaptation to pH variability in purple sea urchins fall in regions with potentially low pleiotropic effects (based on analyses involving regulatory regions, network centrality, and expression time) involved in low pH response (based on functional enrichment).

Keywords: local adaptation, environmental variability, pleiotropy, regulatory variation, sea urchins, high gene flow.

Introduction

One of the major goals of evolutionary biology is to reveal the molecular basis of adaptive evolution in natural populations. Investigating adaptive genetic variation (differences between genomes of individuals as a result of natural selection; Holderegger et al. 2006) can lead to insights into both past and future evolutionary and ecological processes and can help predict species' resilience to future climate conditions (Conover et al. 2006; Bay et al. 2018; Xuereb et al. 2018). One way to study adaptive genetic variation is by examining genetic differences between populations of the same species that experience different selection pressures. Species with broad geographic distributions are commonly distributed across heterogeneous environments, such as discrete habitat patches, or latitudinal and altitudinal gradients, which can result in local adaptation (Savolainen et al. 2007; Schoville et al. 2012), where individuals from a population perform better in their local environment than individuals from nonlocal populations (Savolainen et al. 2013). Local adaptation in response to spatial environmental differences is the result of adaptive genetic differentiation, as some alleles are more beneficial in one environment than others (Kawecki and Ebert 2004; Tiffin and Ross-Ibarra 2014).

High gene flow can hider local adaptation (Slatkin 1987). Still, growing evidence demonstrates the existence of local adaptation between populations experiencing high gene

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flow (Savolainen et al. 2007; Fitzpatrick et al. 2015; Moody et al. 2015; Tigano and Friesen 2016; Hämälä and Savolainen 2019), even in marine species (Sanford and Kelly 2011; Bernatchez 2016). Furthermore, it has been argued that gene flow can promote local adaptation, as it can provide subpopulations with genetic diversity that can facilitate the search for adaptive local phenotypes (Lenormand 2002). By investigating genetic and phenotypic differences between populations with high gene flow, potential molecular mechanisms underlying adaptation to specific environmental conditions can be revealed, as differences between populations are likely to be due to selection rather than drift (Hoban et al. 2016).

The environment organisms experience could vary at both spatial and temporal scales. Interconnected populations that experience differences in temporal variability provide an excellent opportunity to study local adaptation to environmental variability. Different populations experiencing different levels of environmental variabilities can result in the evolution of population-specific phenotypic plasticity if the environmental change is frequent and predictable (Bitter et al. 2021). Indeed, populations experiencing higher temporal variabilities are commonly adapted to have stronger plastic responses (Sanford and Kelly 2011; Pespeni et al. 2013*b*; Franch-Gras et al. 2018; de Villemereuil et al. 2018; Sasaki and Dam 2019).

It is hypothesized that mutations in gene regulatory regions are important in adaptation to environmental variability (Whitehead and Crawford 2006*b*; López-Maury et al. 2008), since differential gene regulation is believed to be one of the main molecular mechanisms underlying phenotypic plasticity (Mäkinen et al. 2018). Furthermore, some studies have found gene regulatory variation to be important for local adaptation in general (Lasky et al. 2014; Gould et al. 2018; Mack et al. 2018; Lewis and Reed 2019). However, few studies have looked at the role of gene regulatory regions in local adaptation to environmental variability. This is largely due to our limited knowledge of noncoding regulatory variation in most species (Poelwijk et al. 2011). To address this gap in knowledge, in this study we present results from a whole-genome population genomics dataset from locally adapted populations experiencing high gene flow and differences in temporal environmental variability.

The purple sea urchin (*Strongylocentrotus purpuratus*) is an excellent model organism for understanding the mechanisms of local adaptation. This species has a wide geographic distribution across a heterogeneous seascape that extends along the west coast of North America, from as far south as Ensenada, Mexico, to as far north as British Columbia, Canada (Ricketts et al. 1985). Purple urchin larvae can travel in the ocean for hundreds of kilometers carried by high-speed currents (Okamoto et al. 2020), which makes sea urchin populations highly interconnected, with little to no fixed allelic differentiation between northern and southern populations (Pespeni et al. 2010; Pespeni and Palumbi 2013). While the mean pH is similar between populations, northern populations generally experience much higher variation in pH. This is due to the upwelling phenomenon in the California Current System caused by seasonal changes in wind direction that pushes the surface of the water offshore and high-dissolved-CO_2, low pH subsurface waters to the surface (Chan et al. 2017).

Low pH conditions significantly affect sea urchin development and physiology, especially at the larval life stage, which largely coincides with the time of the year with the strongest upwelling, April to early May (Chavez et al. 2000). Since the pH of larval extracellular spaces is not regulated, pH has to be maintained by active ion transporters, which is energetically expensive (Stumpp et al. 2012; Evans et al. 2017). Moreover, urchins need carbonate ions to build their skeletons; however, carbonate ions are depleted when the concentration of CO_2 is high (Stumpp et al. 2012). As a result, sea urchin larvae, which develop at low pH, have reduced biomineralization and body size (Pespeni et al. 2013*c*; Chan et al. 2015). Accordingly, transcriptomics studies have found a global downregulation of genes involved in biomineralization and an upregulation of genes involved in ion homeostasis (Evans and Watson-Wynn 2014).

Another advantage of using purple sea urchins to study adaptation to environmental variability is the availability of developmental and genomic resources provided by the echinoderm scientific community (Arshinoff et al. 2022). The reference genome and gene annotations have been recently significantly improved, and with modern high-throughput technologies regulatory sites have been detected genome-wide (DNase-seq, ATAC-seq [Shashikant et al. 2018], Chip-seq [Khor et al. 2019], enhancer RNA [Khor et al. 2021], and noncoding conserved element computational discovery [Tan et al. 2019]). Thus, this study system is ideal to investigate the genetic variation important in local adaptation to environmental variability and to specifically look at the role of mutations in regulatory regions.

In this study, we generated and analyzed the whole-genome sequences of purple sea urchins from seven populations, three of which consistently experience a much higher frequency of low pH events, to investigate adaptive genetic variation and test for signatures of selection to environmental variability. We identify alleles associated with pH variability and determine the types of genomic regions and genes in which these loci lie. We consider the function of these genes as well as the magnitude of their expression during early development and how they relate to one another in a protein-protein interaction network (PPIN).

Methods

Data Collection

We collected, shipped, extracted DNA, and sequenced the whole genomes of 140 purple sea urchins (*Strongylocentrotus purpuratus*), 20 from each of seven sites (fig. 1). Coordinates for collection sites were chosen on the basis of pH data collected by autonomous pH sensors mounted submerged in the water at ecologically relevant depth for this sea urchin species (Evans et al. 2017). Within a site, we limited collections to four or five urchins collected within a 1-m^2 area. Urchins were collected from low intertidal areas and had 4.5–6.0-cm test diameter (the diameter of the hard outer shell of sea urchins). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, and sequencing libraries were prepared using Nextera DNA Flex Small Genomes Library Prep. Paired-end sequencing using NovaSeq S2 Flow Cell 150 × 150 bp on a single lane resulted in high-quality reads, such that no trimming was necessary.

From Raw Reads to Single-Nucleotide Polymorphisms (SNPs)

The Burrows-Wheeler Alignment Tool (BWA) MEM algorithm (Li 2013) was used for mapping the raw reads to the *S. purpuratus* reference genome (Spur ver. 5.0, scaffold N50 ~37 Mbp). The average coverage for each individual

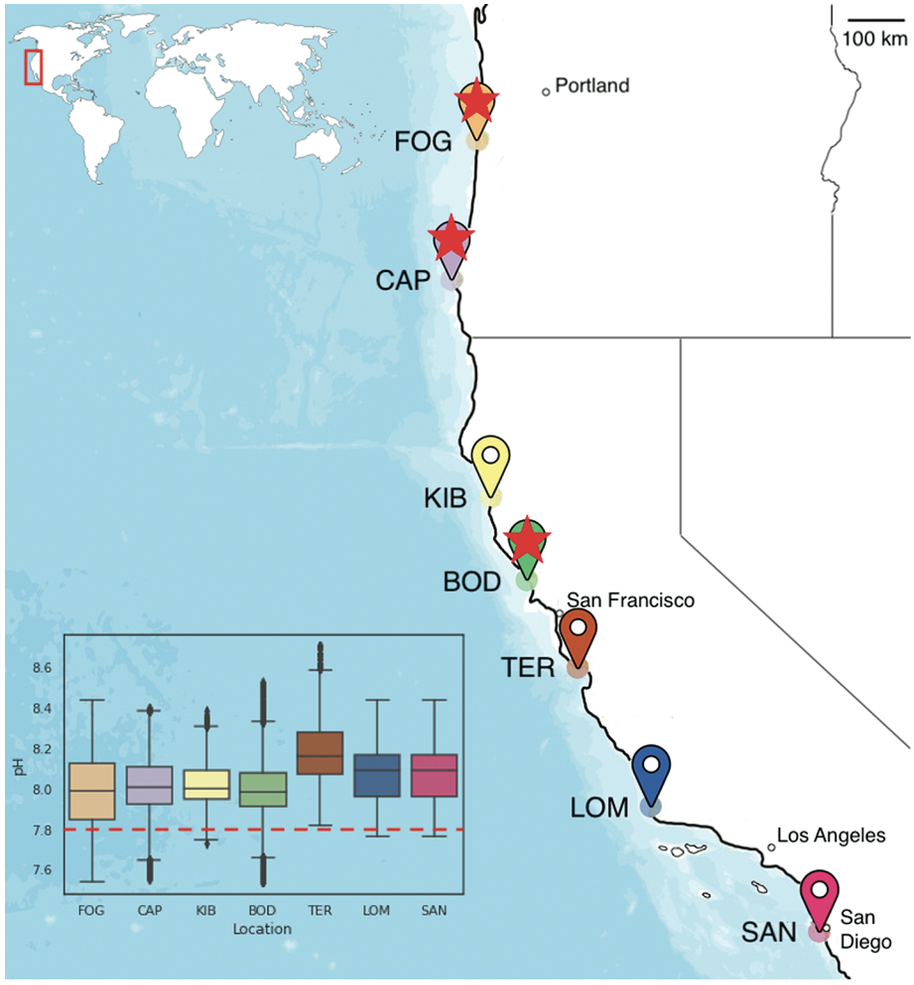

Figure 1: Seven collection sites of adult purple sea urchins (pins), 20 from each location. From north to south: Fogarty Creek (FOG), Cape Blanco (CAP), Kibesilah Hill (KIB), Bodega Head (BOD), Terrace Point (TER), Lompoc Landing (LOM), San Diego (SAN). The inset shows pH data gathered by autonomous pH sensors at those locations across all years and months for which the sensors were deployed (2011, 2012, and 2013, April to October). Individuals were collected in June and July 2020, within 500 m of where the pH data were measured (Evans et al. 2017), except for SAN, for which pH data were estimated on the basis of the nearest IPACOA buoy. The red line indicates pH 7.8, below which only FOG, CAP, and BOD had frequent measurements (sites indicated with red stars on map).

was 6.42 ± 0.78, with an average mapping rate of 81.6 ± 0.01. The Analysis of Next Generation Sequence Data (ANGSD) program was run to calculate genotype likelihoods of polymorphic sites across all populations (maximum *P* value: 1e−6; Korneliussen et al. 2014). The output beagle file was then used by PCAngsd to create a covariance matrix to visualize any clustering by population and environmental factors, as well as coverage. There was no clustering present, showing that there were no differences in sequencing success between individuals and populations (Meisner and Albrechtsen 2018). Based on the principal component analysis (PCA), three outlier individuals were removed from further analysis (two from Fogarty Creek and one from Cape Blanco).

Next, ANGSD was used to produce a variant call file (vcf) for all high-quality polymorphic sites across all individuals filtered by sequencing read, depth, and mapping quality, yielding 15,902,843 polymorphic sites. The vcf file was then further filtered, leaving 994,220 biallelic SNPs with a minimum minor allele frequency (MAF) of 0.025 to use in downstream analyses. ANGSD was also run separately for each population, this time for all sites, not just for significantly polymorphic ones. The resulting seven site allele frequency (saf) files were then used by realSFS (part of the ANGSD software; methods based on Korneliussen et al. 2013) to calculate all 21 pairwise site frequency spectrums (sfs) followed by per site as well as global F_{ST} values. The output of realSFS was also used to calculate per-site nucleotide diversity measures by thetaStat (window size = 1,000, step size = 1,000). PLINK version 1.9 was used to estimate linkage disequilibrium (Purcell et al. 2007).

Identification of pH Variability–Associated SNPs

pH data from Evans et al. (2017) was used to group populations on the basis of frequency of low pH events. pH measures (2011, 2012, and 2013, April to October) had similar means across the populations. However, while four of the seven populations experience low pH events only occasionally, three populations (Bodega Head, Cape Blanco, and Fogarty Creek) experience physiologically relevant low pH conditions often (fig. 1, *inset*). When looking at the frequency of pH events <7.8 (<2% vs. ~10% of all data points) as well as absolute minimum pH measured (~7.75 vs. ~7.5), lowest 100 pH measures averaged (~7.8 vs. ~7.6), or lowest first percentile of pH data (~7.8 vs. ~7.65), these populations clearly partition into the above-mentioned two groups.

We identified SNPs where the allele frequency strongly correlated with the frequency of low pH conditions (as a categorical environmental variable: high or low frequency of low pH) using three independent methods: OutFLANK, pairwise F_{ST}, and LFMM2. With OutFLANK, we looked for allele frequency differences based on regrouping of all individuals into the above-mentioned two categories (i.e., considering two populations instead of seven). We required a minimum heterozygosity of 0.1 with a *q*-value of 0.05, with default settings (Whitlock and Lotterhos 2015). Pairwise per-site F_{ST} values were instead calculated by averaging between populations experiencing similar frequency of low pH conditions (within-category average F_{ST} [*W*]) as well as between populations belonging to different categories (between-category average F_{ST} [*B*]), and then the within-category average was subtracted from the between-category average ($B - W$) to find genomic sites where on average the F_{ST} between populations experiencing low and high frequency of low pH was the greatest compared with the average F_{ST} between populations of the same category. Outliers (highest 1% F_{ST} values) were selected on the basis of a standard method of bootstrapping, which involved randomly sampling the distribution of F_{ST} values with replacement and calculating the 99th percentile of the new distribution 10,000 times. To get the bootstrapped cutoff value for the top 1%, the 95th percentile of these 10,000 percentiles was taken. Finally, LFMM2, a method commonly used to infer gene-environment associations using latent factor mixed models, was run using ridge estimates and $k = 1$ because of the lack of population structure shown by both LFMM2 and PCAngsd PCAs (Caye et al. 2019). Since the results from these three methods were significantly positively correlated (fig. S2), we focused on only one of these methods, and the results presented below were calculated on the basis of the pairwise F_{ST} measures (fig. S3).

From Candidate SNPs to Genomic Regions and Genes

SNPs were annotated using the Spur (ver. 5.0) annotation available on NCBI RefSeq (PRJNA10736 BioProject accession number) as well as the Variant Effect Predictor available on EnsemblMetazoa (McLaren et al. 2016). Regions within 5,000 bp upstream of the transcription start site for each gene were considered as promoter regions in our analysis (results were unaffected by considering promoters as 2,000 bp upstream). Putative enhancers were identified by combining the following previous published datasets: ATAC-seq and DNA-seq overlaps (Shashikant et al. 2018), Chip-seq (Khor et al. 2019), conserved noncoding elements with *Lytechinus variegatus* (Tan et al. 2019), experimentally verified enhancers (Nam et al. 2010; Arenas-Mena et al. 2021), and enhancer RNAs (Khor et al. 2021). An outlier SNP was considered an enhancer SNP if it fell in a noncoding region in addition to a region included in one of the above-mentioned datasets. Additionally, long noncoding RNA (lncRNA) regions were identified on the basis

of computational prediction present in the NCBI RefSeq annotation file (gff-version 3) as well as based on a comparative study by Hezroni et al. (2015). A χ^2 test was used to check whether the distribution of F_{ST} outliers between different genomic regions was significantly different from expectation. If a SNP fell in more than one region, all regions were counted (randomly choosing one did not affect results).

Gene Expression

Early developmental gene expression data (within 72 h postfertilization [hpf]) was downloaded from the Echinobase FTP (Arshinoff et al. 2022). Genes were categorized as "highly expressed during early development" if the sum of transcripts per million over all time steps was greater than 700 (top 5% of data; determined on the basis of the shape of the distribution, the point at which increasing the threshold would result in exponential loss of data). A binomial statistical test (SciPy package; Virtanen et al. 2020) was used to test for enrichment of genes highly expressed during early development among genes with an outlier SNP.

Protein-Protein Interaction Network

The PPIN for *S. purpuratus* including only physical links (either experimentally determined or information gathered from curated databases) was downloaded from the String database (Szklarczyk et al. 2020). The network of size 20,180 was pruned on the basis of the confidence score of the edges (threshold = 750), leaving 5,901 nodes (i.e., proteins). For genes with and without outlier SNPs, both degree centrality (number of neighbors) and betweenness centrality (the number of times the node of interest is on the shortest path between two other nodes) was determined using networkx (Hagberg et al. 2008). A Mann-Whitney *U* test was used to compare these measures between the two groups. For network visualization and modularity calculation, Gephi software (ver. 0.9) was used with the Fruchterman Reingold layout (area = 10,000, gravity = 10, speed = 1; Bastian et al. 2009).

Functional Enrichment

Gene Ontology (GO) terms for each gene with one or more outlier SNPs in the promoter or gene body region were accessed from UniProt (UniProt Consortium 2021). To test for functional enrichment, we used the latest version of topGO (Alexa and Rahnenführer 2022). In addition, the set of genes with one or more outlier SNPs was tested for significant overlap with published sets of genes, including a previously identified list of biomineralization genes (Evans et al. 2017) and genes with SNPs identified as changing in allele frequency in response to low pH conditions in single-generation selection experiments (Brennan et al. 2019). Again, significance was tested using a binomial test (SciPy package; Virtanen et al. 2020).

To further investigate variation in biomineralization genes, a neighbor-joining tree visualizing the hierarchical relationship between the populations based on the pairwise differences of all filtered SNPs (not just outlier SNPs) falling in biomineralization genes was made. Pairwise differences between each pair of individuals (all 137) were computed by counting the number of sites they differed out of the ~1 million filtered SNPs. Then these measures were averaged to calculate a similarity between each pair of populations. These steps were repeated for all filtered (including nonoutlier) SNPs as a base of comparison. Neighbor-joining trees were generated using MEGA11 software, a neighbor-joining algorithm, and then visualized using FigTree (ver. 1.4.4).

Results

Global Patterns across Populations and the Whole Genome

The genomes of the sequenced sea urchins were highly polymorphic. Considering the genomes of all 140 individuals, we identified ~16 million high-quality SNPs and estimated an overall pairwise nucleotide diversity measure (π) of 0.0254.

PCA showed no clustering by population, geographic region (north vs. south), or frequency of low pH events (low vs. high) when looking across the first five principal components (fig. 2). Results were the same when the analysis was repeated with only SNPs in gene bodies or exonic regions. We also found no evidence for isolation by distance when comparing pairwise global F_{ST} measures to geographic distance ($r^2 = 0.009$, $P = .969$). Furthermore, we found very low linkage disequilibrium among the genomic positions (1,500 bp; fig. S1).

Distribution of SNPs Involved in Adaptation to pH Variability

The further-filtered high-quality SNPs (MAF > 0.025 and biallelic across all individuals; ~1 million) were distributed evenly across all large scaffolds in the reference genome (fig. 3*A*). Considering different classes of genomic regions, the bootstrapped 1% highest F_{ST} SNPs between regions of low and high pH variability (9,780 outlier SNPs) were nonrandomly distributed among genomic regions (χ^2 test, $P <$ .001). Specifically, although there were fewer intronic outlier SNPs, there were more promoter, untranslated region (UTR), and exonic positions with high F_{ST} than expected,

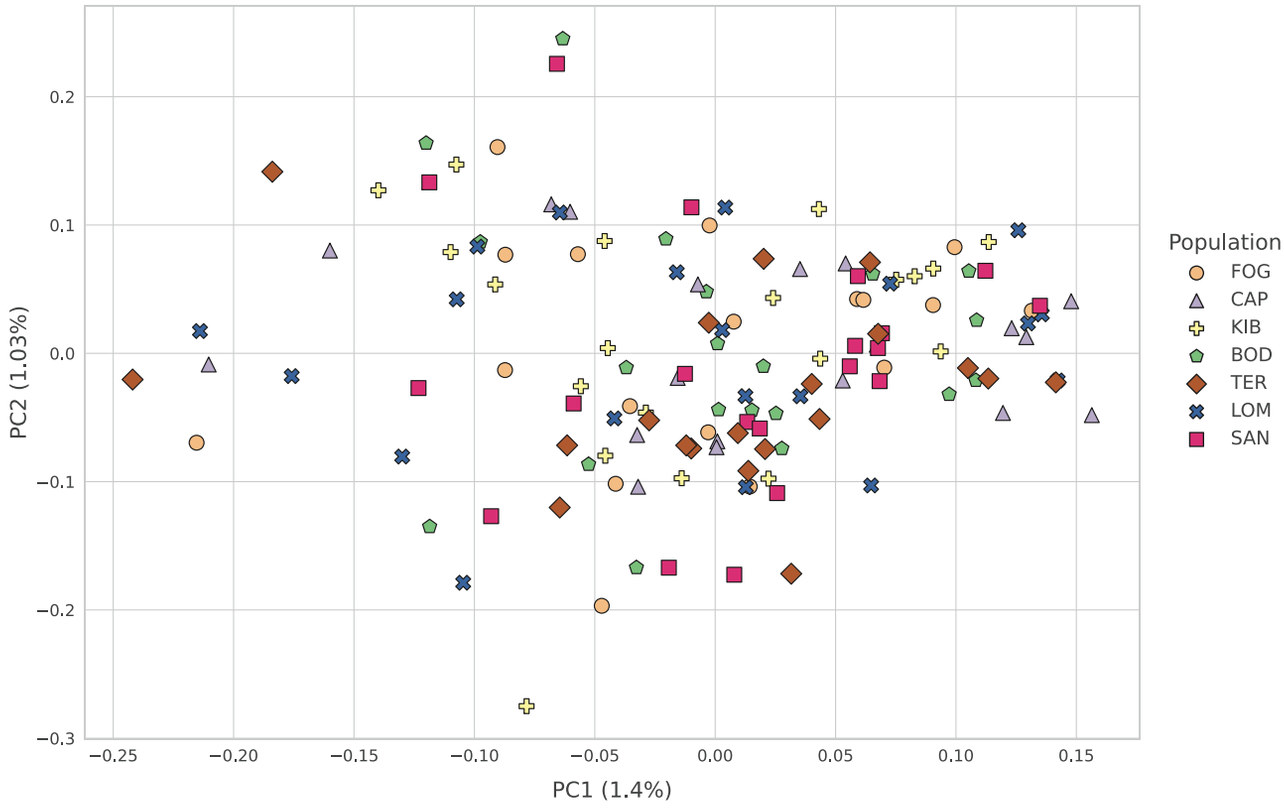

Figure 2: Principal component analysis of genome-wide variation across all high-quality single-nucleotide polymorphisms identified among 137 individuals from the seven populations (three outlier individuals were dropped). Populations in the key are ordered from north to south (codes are as in fig. 1), and the colors of the shapes correspond to the colors on the map in figure 1.

especially in putative and experimentally confirmed noncoding enhancer and lncRNA regions (fig. 3*B*). In addition, regardless of genomic location (i.e., including coding regions), we found 99 outliers in enhancer RNAs (vs. 60 expected; $P < .0001$) and 13 outliers in ChIP-seq-identified regulatory regions (vs. 7.65 expected; $P < .0485$). Considering outliers in exonic regions, a smaller percentage of exonic F_{ST} outliers were nonsynonymous mutations than expected (25.2% vs. 32.1%; $P < .0001$), while a greater percentage of exonic F_{ST} outliers were synonymous mutations than expected (74.7% vs. 67.8%; $P < .0001$). We found as many stop-loss and stop-gain variants as expected by chance (1 for each).

Types of Genes Associated with pH Variability

To understand the potential functional consequences of outlier SNPs, we (1) tested the prevalence of genes with outlier SNP(s) among genes expressed highly during early development, (2) investigated the network position of genes with outliers in the PPIN, and (3) conducted a functional enrichment analysis. Considering gene expression during early development, we found that genes with outlier SNPs in the promoter region were significantly underrepresented among early developmental genes highly expressed within 24 ($P = .0249$), 48 ($P = .00609$), and 72 ($P = .0294$) hpf (fig. 4). On the other hand, genes with outlier SNPs in gene bodies were not under- or overrepresented among genes highly expressed during early development (fig. 4; <24 hpf, $P = .15$; <48 hpf, $P = .67$; <72 hpf, $P = .93$). When looking at outlier SNPs specifically in exonic regions of these genes, they were overwhelmingly synonymous (90.3% vs. 67.8% expected; $P < .0001$). Comparing genes with outlier SNPs regardless of position in promoter or gene body regions to all early development genes, we found significant underrepresentation of outliers among genes highly expressed within 24 hpf ($P = .03$) but not beyond that (<48 hpf, $P = .23$; <72 hpf, $P = .68$). Taken together, these results suggest conservation of genes expressed early in development, particularly in the promoter regions.

To explore the physical interactions of genes with outlier SNPs, we used network analyses of a PPIN. Genes with outlier SNPs were on average less connected in the

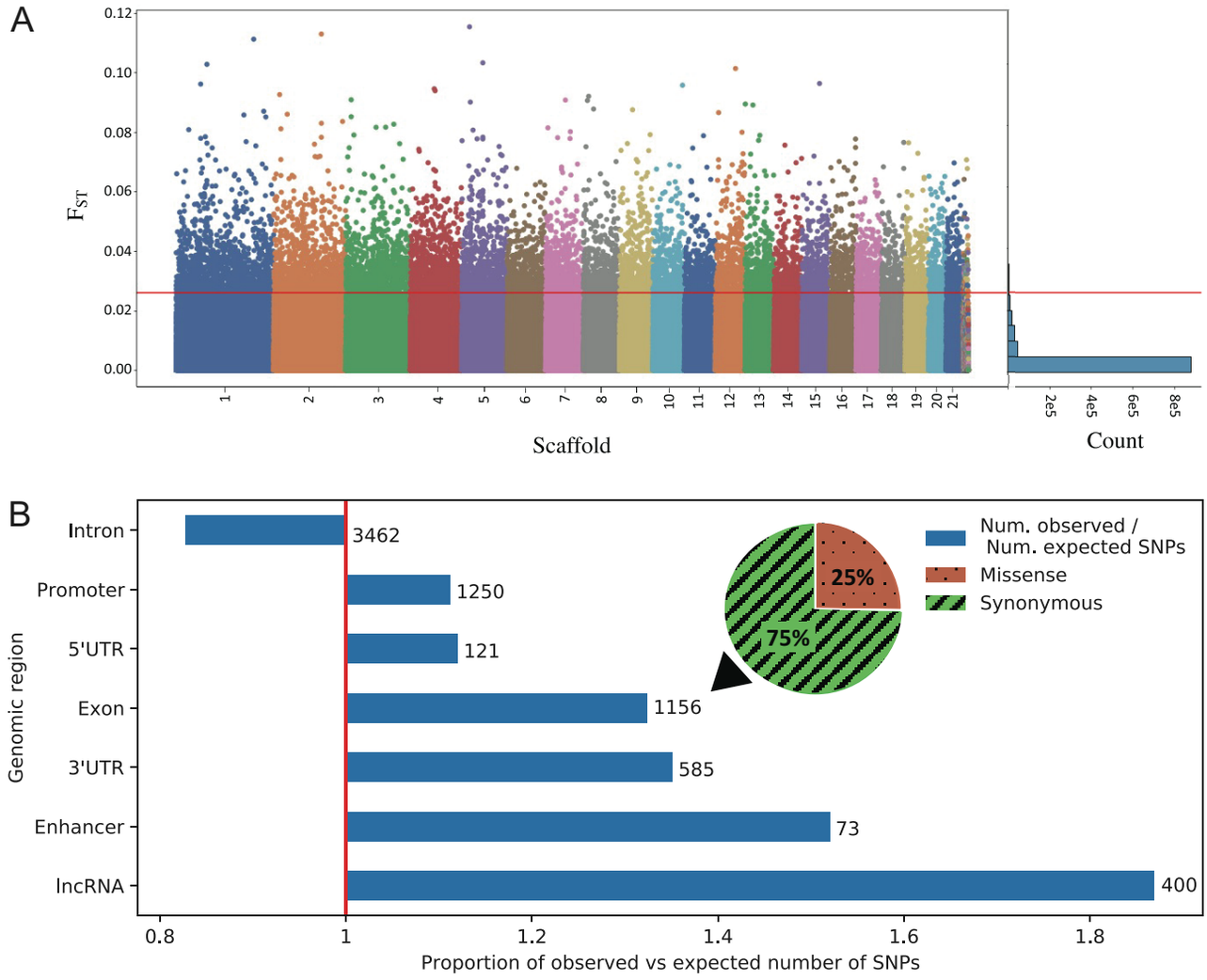

Figure 3: Genome-wide patterns of variation. *A*, Manhattan plot of F_{ST} values between low and high pH variability populations (average between-group F_{ST} minus average within-group F_{ST}) calculated for all ~1 million filtered single-nucleotide polymorphisms (SNPs), colored on the basis of scaffold. Scaffolds are numbered and ordered on the basis of the number of SNPs they have, most to least. The red line shows the cutoff value for bootstrapped top 1% highest F_{ST} values (i.e., outliers). The rotated histogram on the right shows a histogram of F_{ST} values. *B*, Proportion of observed versus expected number of outlier SNPs in each genomic region. The overall observed distribution is significantly different than expected (χ^2 test, $P < .001$), and testing each category separately yields $P < .05$ for all categories except 5′ untranslated region (UTR). Intronic regions were the only regions significantly underrepresented. Numbers on the right indicate the number of outlier SNPs found in that region, out of 9,780. The pie chart shows the percentage of outlier SNPs in exonic regions that were nonsynonymous and synonymous.

PPIN than other genes, both considering outliers in promoter regions and in gene bodies (i.e., degree centrality, average number of interacting partners 10.14 vs. 20 [$P < .0001$] and 12.19 vs. 20 [$P < .0001$], respectively; fig. 5*A*). In addition, genes with outliers had on average a lower betweenness centrality compared with other genes ($P < .0001$; again for both genes with outliers in promoter and gene bodies), meaning that genes with outliers not only had fewer neighbors in the network, they were also not key nodes on the shortest path between two other nodes (fig. 5*B*). Additionally, we found that while genes with outlier SNPs were less connected on average, there was a significant PPIN enrichment (based on the String database enrichment algorithm; Szklarczyk et al. 2020), suggesting that genes with outlier SNPs were more interconnected with each other than expected compared with a random set of genes of the same size and degree distribution drawn from the genome ($P = .0002$), indicating that these genes

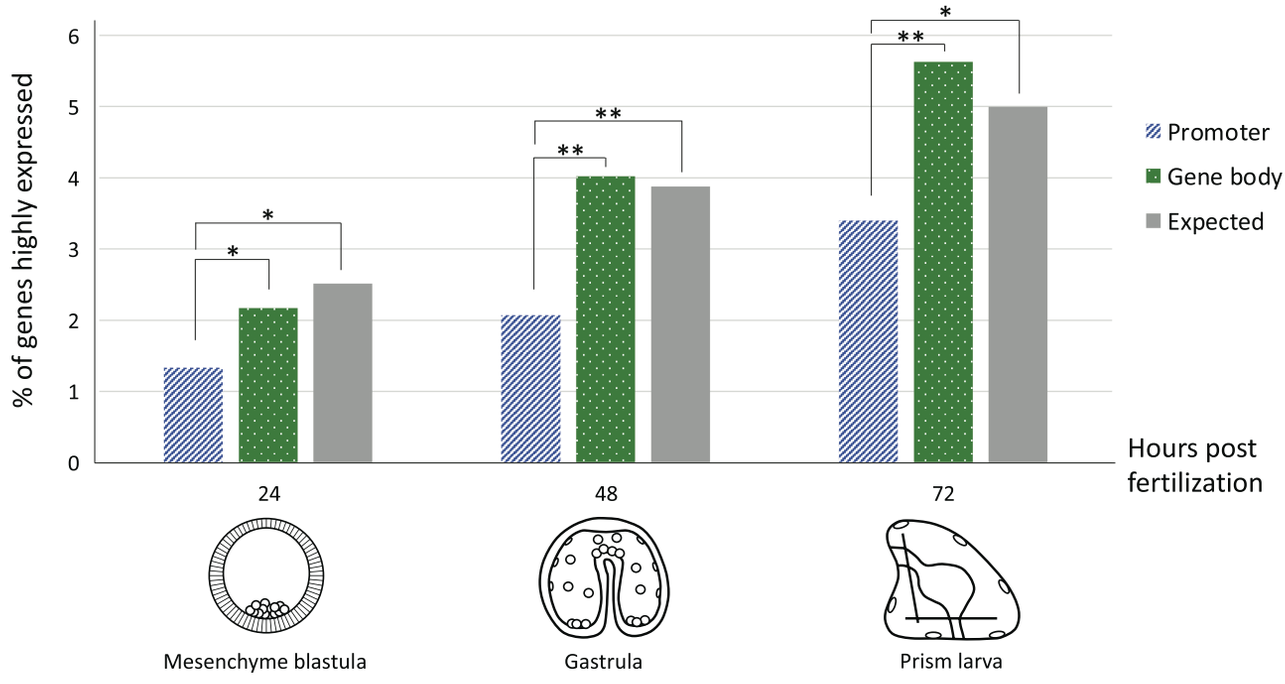

Figure 4: Percentage of genes with outlier single-nucleotide polymorphisms (SNPs) in promoter (blue, striped) or gene body (green, dotted) regions that are highly expressed at distinct stages of embryonic development compared with the expected percentage (i.e., percentage of genes highly expressed out of all genes). Genes with outlier SNPs in their promoter regions were significantly underrepresented at all stages of early development.

of interest share biological functions, developmental timing of expression, and/or cell types in which they are expressed (fig. 5*B*).

Last, genes with either gene body or promoter outlier SNPs were enriched for 42 biological process GO categories, including cell surface receptor signaling, cell adhesion, and ion transmembrane transport, as well as 55 molecular function GO categories, including ion gated channel activity, calcium-activated cation channel activity, and carbonate dehydratase activity (table S1). Considering the list of previously identified biomineralization-related genes, we found more biomineralization genes with either gene body or promoter outlier SNPs than expected (77 observed vs. 60.9 expected; χ^2 test, $P = .02529$), with the pattern being driven by gene body outliers.

To further test the importance of biomineralization genes in adaptation to the frequency of low pH using a method independent of finding outlier SNPs, we built a neighbor-joining tree of the populations using all SNPs across the genome (994,220 SNPs) and all SNPs that fall in biomineralization genes (12,576 SNPs). We found that when only considering SNPs in biomineralization genes, the populations clustered into pairs on the neighbor-joining tree on the basis of the frequency of low pH the populations experience, in contrast to considering all SNPs (fig. 6*A*, 6*B*). Specifically, we found that Cape Blanco and Fogarty Creek, populations experiencing low pH conditions most frequently, and Kibesilah Hill and Terrace Point, populations experiencing a similar low frequency of low pH conditions, were most similar to each other. As expected, when we repeated the same analysis using only SNPs in biomineralization genes that were classified as outliers, clustering of populations by pH variability becomes even stronger (fig. 6*C*).

Discussion

In this study, we set out to test for signatures of local adaptation to environmental variability in whole-genome sequence data. While looking across the entire genomes, we found no evidence for population structure (fig. 2); individuals collected 1,700 km away were not more genetically different from individuals collected just a few meters away. Despite a lack of isolation by distance or any kind of clustering by population or geographical region, we found SNPs strongly correlated with pH variability. Enhancers and lncRNA were particularly enriched for loci under selection (fig. 3*B*). Furthermore, we confirmed previous results and found that promoter and gene body loci under selection were enriched in genes involved in

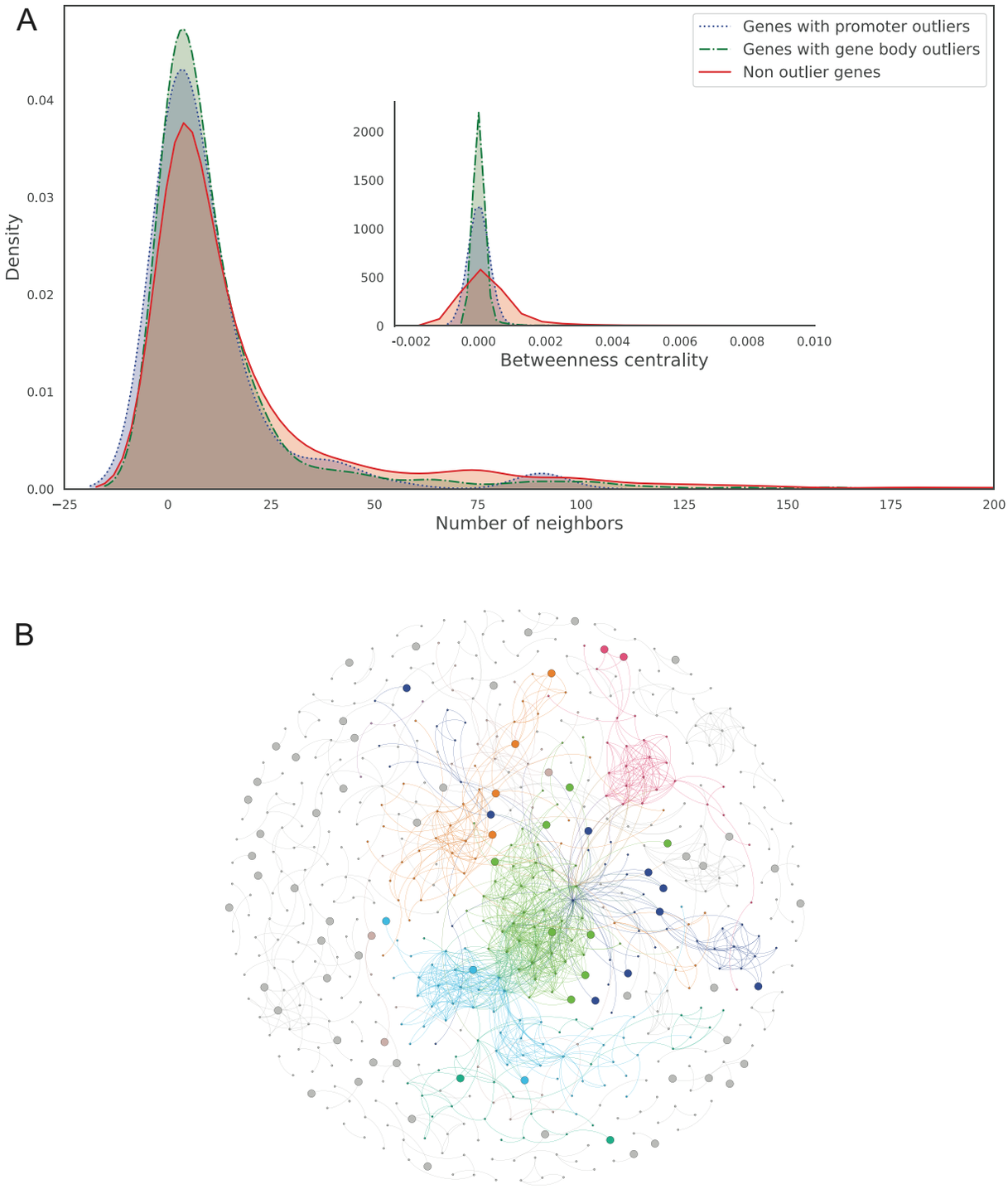

Figure 5: *A*, Density plot of the number of neighbors and betweenness centrality in the protein-protein interaction network (blue = outlier single-nucleotide polymorphism [SNP] in the promoter region; green = outlier SNP in the gene body; red = no outlier SNPs in gene). Maximum numbers are not shown: 385 and 0.118 for number of neighbors and betweenness, respectively. Proteins with no outlier SNPs had on average a higher degree centrality and betweenness centrality. *B*, Network visualization of a random subset of 1,000 nodes. Colors correspond to different modularity classes, and larger nodes represent proteins with outlier SNP(s) in either promoter or gene body regions. Only nodes with a minimum of one edge are shown.

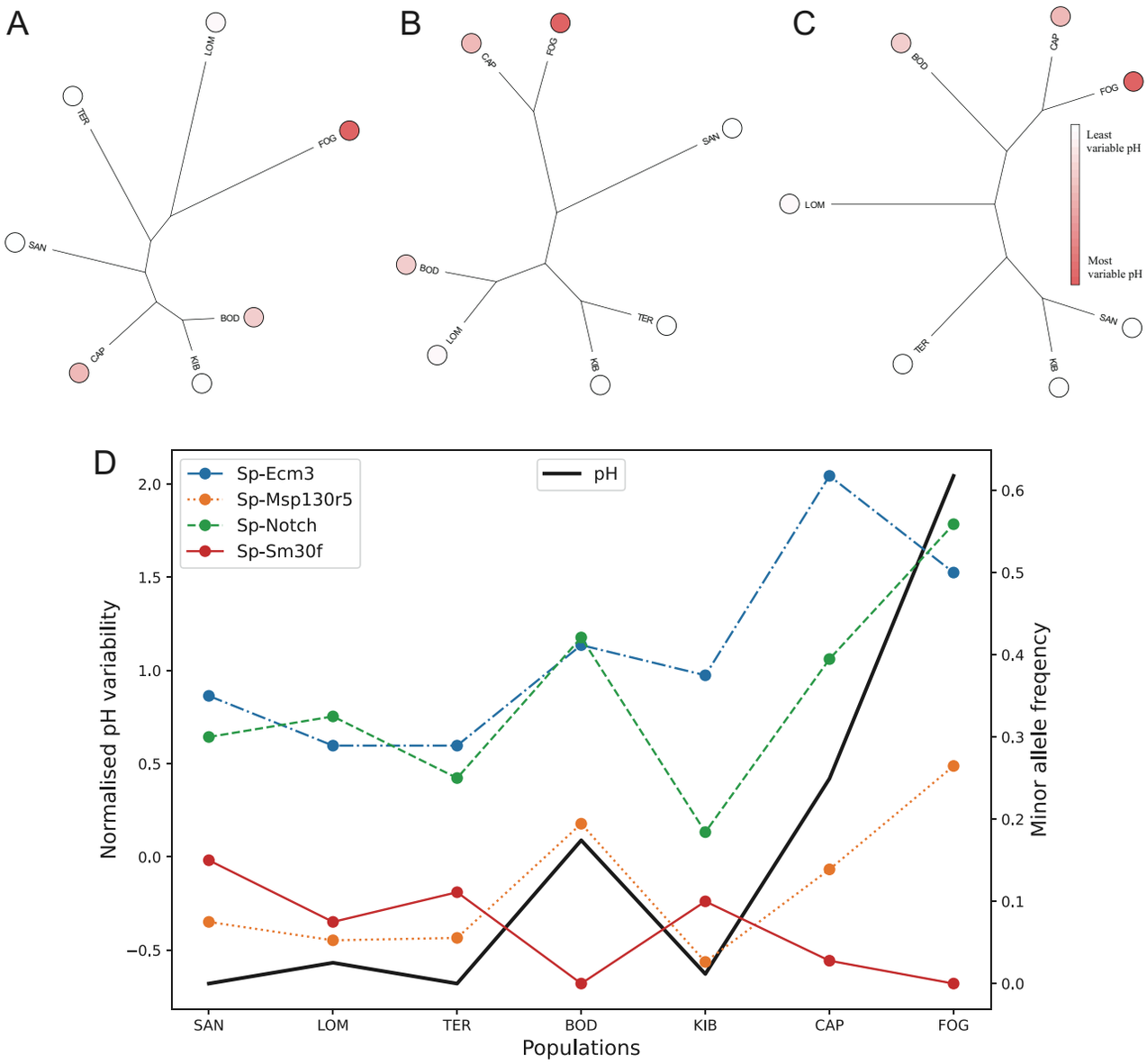

Figure 6: Neighbor-joining tree calculated considering all single-nucleotide polymorphisms (SNPs; no clustering; *A*), all SNPs in biomineralization genes (significant clustering; *B*), and only outlier SNPs in biomineralization genes (perfect clustering; *C*). The color of nodes indicates the normalized frequency of pH less than 7.8. *D*, Four examples of outlier SNPs in different biomineralization genes that correlate with frequency of pH under 7.8. Colored lines show allele frequencies, and the black line shows the frequency of pH under 7.8.

biomineralization and ion homeostasis (Pespeni et al. 2013*b*; Evans et al. 2017). We also found that genes with outlier loci were underrepresented among genes expressed early in development (fig. 4) and that they were generally less connected in the physical PPIN (fig. 5). We hypothesize that all of these results together suggest that loci with low pleiotropic effects are important fuel for local adaptation to environmental variability.

High gene flow between populations can hinder local adaptation in that differences in genetic composition between populations can be reduced by the constant exchange of alleles due to migration, in the case of purple sea urchins, during the larval stage of development (Endler 1977; Slatkin 1987; Galindo et al. 2010; Pespeni et al. 2012, 2013*a*). Indeed, in accordance with previous studies involving this species (Pespeni et al. 2010), we found little overall genetic differentiation between the populations under investigation (fig. 2). Despite the lack of a global population structure, we found a suite of loci correlated with pH variability. This apparent paradox between high

gene flow but population-specific differences at specific loci could be explained by the high genetic diversity ($\pi = 0.0254$) across all of the sequenced individuals in combination with a low linkage disequilibrium (fig. S1). The combination of high genetic diversity and low linkage disequilibrium enables populations to have a diverse set of alleles on various genomic backgrounds. These alleles can then be subject to selection in different ecological niches (Levene 1953; Yeaman and Jarvis 2006). In addition, having high gene flow in the study system has its advantages. First, any consistent difference between populations experiencing low and high pH variability are more likely to be due to selection rather than drift; and second, high gene flow together with high fecundity and large population size maximize the effects of natural selection (Palumbi 1992; Hartl et al. 1997). Here, we tested for pH-associated loci, identified the genomic regions where they most often occur, and explored how they may relate to adaptive phenotypes by integrating with other sources of genomic data.

The Importance of Regulatory Regions

In this study, we found an overrepresentation of outlier SNPs in regulatory regions. Regulatory regions are hypothesized to be involved in local adaptation to environmental variability in this species for two main reasons. First, highly interconnected populations of purple sea urchins have been experiencing different pH variabilities due to upwelling for about 10 million years (Jacobs et al. 2004), when they radiated to this region of the Pacific Ocean from the arctic southward (Biermann et al. 2003), giving the populations plenty of time to adapt. Because of these differences in variability, it is expected that individuals from different populations would have different plastic responses in response to low pH. Indeed, previous studies have found that transcriptional response to low pH and consequent phenotypic changes of this species is population specific. Larvae from southern populations were found to have a lower level of expression of genes within major ATP-producing pathways and a larger decrease in body size when exposed to low pH (Kelly et al. 2013; Evans et al. 2017), and adults of the same population after 3 years of common garden conditions had lower expressions of biomineralization genes and a faster spine regrowth rate than their northern counterparts (Pespeni et al. 2013*a*), indicating the presence of local adaptation to pH variability through differences in environmentally responsive as well as constitutive gene expression. Genetic differences in regulatory regions between the populations investigated in this study (fig. 3) could underlie the gene expression differences found by previous studies.

Second, the importance of variation in cis-regulatory elements that control the expression of nearby genes in driving local adaptation is increasingly recognized now that whole-genome sequencing is more affordable and genomes are better characterized (Juneja et al. 2016; Joshi et al. 2021). Many studies have found locally adapted natural populations to have different gene expression patterns, including research on fruit flies (Levine et al. 2011; Zarubin et al. 2020), mummichog and gulf killifish (Oleksiak et al. 2002; Whitehead and Crawford 2006*a*; Brennan et al. 2015), and valley oak (Gugger et al. 2017). However, only recently have we been able to find the genomic regions underlying these differences through genome-wide scans with studies primarily of human local adaptation. SNPs influencing gene expression were identified as targets of recent natural selection contributing to human local adaptation (Kudaravalli et al. 2009), regulatory regions were found to have many signatures of local adaptation (Vernot et al. 2012), and Fraser (2013) showed that loci involved in local adaptations are much more likely to affect gene expression than protein structure (Fraser 2013). Additionally, regulatory variation was found to play a significant role in local adaptation in natural populations of *Arabidopsis thaliana* (Lasky et al. 2014), yellow monkeyflower (Gould et al. 2018), house mice (Mack et al. 2018), stickleback fish (Jones et al. 2012), and *Heliconius erato* (Lewis and Reed 2019), with a counterexample of a lack of excess of cis-regulatory variation associated with short-term response to strong selection pressures in the grain crop millet (Rhoné et al. 2017). Mutations in gene regulatory regions have also been found to be important in adaptation to environmental variability (Whitehead and Crawford 2006*b*; López-Maury et al. 2008), in line with the hypothesis that differential gene regulation is one of the main molecular mechanisms underlying phenotypic plasticity (Mäkinen et al. 2018).

In addition, outliers in coding regions may also relate to regulation. We found an excess of synonymous mutations among SNPs involved in adaptation to pH variability. Recent findings highlight the potentially important phenotypic effects of synonymous mutations, including translation efficiency, messenger RNA stability, and recognition by small regulatory RNAs (Kristofich et al. 2018). Thus, similar to mutations in promoter or enhancer regions, these mutations could influence the regulation of protein expression, but on the translation level. However, further research is needed to investigate the effect of synonymous mutations and their potential importance in local adaptation.

The Pleiotropic Effects of Loci Involved in Adaptation

Cis-regulatory mutations are predicted to play a large role in local adaptation because these mutations are likely to have fewer deleterious pleiotropic effects than mutations causing changes to amino acid sequences (Stern and Orgogozo 2008). While changes in protein structures can

have many downstream consequences in protein-protein interactions, signaling pathways, and enzymatic reactions, variation in cis-regulatory elements often only influence the level of expression of a particular gene, in a specific tissue, at a certain developmental stage or in response to a distinct external stimulus (Hoekstra and Coyne 2007) and are likely an import fuel to local adaptation as they enable fine-tuning of gene expression with lower fitness costs (Prud'homme et al. 2007). While this hypothesis is hard to test experimentally, a recent study did find strong evidence that cis-regulatory mutations have smaller phenotypic and fitness consequences than trans-regulatory mutations in yeast (Vande Zande et al. 2022).

The hypothesis of reduced pleiotropic effect can also be used to interpret our result that we found fewer genes with signatures of local adaptation in their promoter that are highly expressed during early development than expected (fig. 4). Mutations in genes expressed highly during early development could have large phenotypic effects, in part because they are likely located in the highest levels of the embryonic development gene regulatory network and thus could have large effects on downstream gene expression patterns and development (Yu and Gerstein 2006). Not surprisingly, these genes are generally highly conserved (Erwin and Davidson 2009). In addition, there is a negative relationship between expression level and the evolutionary rate of a protein, although the reason for this remains largely unknown (Drummond et al. 2005; Zhang and Yang 2015).

Differential pleiotropic effects are also a reason why proteins less central in a protein-protein interaction, gene coexpression, or gene regulatory network are likely the ones involved in adaptation, based on both early theory (Fisher 1930) and recent experimental results. Because central genes/protein are under strong selective constraint (Fraser et al. 2002; Hahn and Kern 2005; Mähler et al. 2017; Masalia et al. 2017), many studies found signatures of long-term positive selection in less connected nodes of biological networks (Kim et al. 2007; Josephs et al. 2017). This is likely because mutations in genes with a higher number of connections have larger pleiotropic effects (He and Zhang 2006; Alvarez-Ponce et al. 2017), and thus less central genes are less constrained to adapt (Stern and Orgogozo 2008). In populations that are well adapted to their environment, small-effect loci are more likely to be used by positive selection and therefore increase in frequency over time (Orr 1998). On the other hand, if a population is further away from the fitness optima—for example, because of recent environmental change—more central positions with higher pleiotropic effects could be involved in adaptation. Indeed, this theoretical prediction was observed in nature, in previous studies involving common ragweed (Hämälä et al. 2020), and human populations (Luisi et al. 2015). Because purple sea urchins have experienced consistent ranges of pH variability throughout their evolutionary history (Jacobs et al. 2004), we predicted that loci involved in local adaptation would have small-effect sizes and be more on the periphery of the PPIN. Indeed, this is what we found. Centrality, computed as both degree and betweenness centrality, was significantly lower for genes with allelic differences between the populations investigated in this study (fig. 5).

During our analysis of the PPIN, we also found that while genes with signatures of local adaptation were on average less connected, they were not randomly distributed across the network, as they were more connected to each other than expected by chance, indicating that they are involved in similar biological functions and cell types. Indeed, functional enrichment analysis showed that many GO categories, including ion transmembrane transport as well as carbonate dehydratase activity, were significantly enriched, and we also found more outliers in biomineralization genes than expected (fig. 6). These results are in line with results of several previous studies that used restriction site tiling or exome or transcriptome sequencing to find signatures of local adaptation to low pH among these populations (Pespeni et al. 2010, 2013*b*, 2013*c*; Evans et al. 2017). Also, when looking only at genes with outlier SNPs in gene bodies, we found that they significantly overlap with genes that were previously found to be under selection in a single-generation selection experiment in response to pH 7.5 ($P = .035$; Brennan et al. 2019).

Conclusion

In conclusion, we found that purple sea urchin populations experiencing different environmental conditions across both space and time have remarkably high genetic variation. Interestingly, variation putatively involved in local adaptation to temporal environmental variability, specifically variability in low pH conditions, was found to be in regulatory regions, proteins less connected in the PPIN, and lacking in genes expressed highly during early development. These results suggest that sites with lower pleiotropic effects are involved in local adaptation to environmental variability in the populations under investigation and perhaps in general in populations of species close to their fitness peaks. Functionally, as expected on the basis of the physiological effects of low pH in sea urchins, adaptive variation was found in genes involved in ion homeostasis and biomineralization. Taken together these findings indicate the presence of high adaptive genetic variation in this species that likely translates into adaptive genetic variation underlying diversity in plastic responses to low pH conditions found by previous studies (Pespeni et al. 2013*a*; Evans et al. 2017). Since diversity in the plastic response itself is crucial for resilience to sudden environmental changes

(Oostra et al. 2018), these populations have the potential to adapt to increased ocean acidification resulting from global climate change; however, genetic variation could become depleted in the process of rapid adaptation (Lloyd et al. 2016; Lin et .al. 2023).

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Statement of Authorship

C.P. and M.H.P conceptualized the study and developed the sampling design. C.P. conducted laboratory work. C.P., L.F., and R.S.B. contributed to the data collection (sequencing and raw data processing). C.P., L.F., R.S.B., and M.H.P. contributed to data analysis, visualization, and interpretation. C.P. led the writing under the supervision of M.H.P. All authors contributed to editing the article.

Data and Code Availability

All code to replicate data processing and analyses can be found on Zenodo (https://zenodo.org/badge/latestdoi/542644288; Petak et al. 2023) and GitHub (https://github.com/PespeniLab/urchin_local_adapt_WGS). Sequence data are available in the National Center for Biotechnology Information Sequence Read Archive (BioProject accession number PRJNA674711.

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