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The genome-wide signature of short-term temporal selection

Michael Lynch[a,1], Wen Wei[a], Zhiqiang Ye[b], and Michael Pfrender[c]

Affiliations are included on p. 9.

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Despite evolutionary biology's obsession with natural selection, few studies have evaluated multigenerational series of patterns of selection on a genome-wide scale in natural populations. Here, we report on a 10-y population-genomic survey of the microcrustacean *Daphnia pulex*. The genome sequences of $>$800 isolates provide insights into patterns of selection that cannot be obtained from long-term molecular-evolution studies, including the following: the pervasiveness of near quasi-neutrality across the genome (mean net selection coefficients near zero, but with significant temporal variance about the mean, and little evidence of positive covariance of selection across time intervals); the preponderance of weak positive selection operating on minor alleles; and a genome-wide distribution of numerous small linkage islands of observable selection influencing levels of nucleotide diversity. These results suggest that interannual fluctuating selection is a major determinant of standing levels of variation in natural populations, challenge the conventional paradigm for interpreting patterns of nucleotide diversity and divergence, and motivate the need for the further development of theoretical expressions for the interpretation of population-genomic data.

population genomics | *Daphnia* | fluctuating selection | molecular evolution | temporal variation

Significance

Except for mono/oligogenic traits known to be under strong selection, there is little information about the temporal dynamics of allele-frequency changes in well-defined and unmanipulated natural populations. A multiyear, genome-wide analysis of nearly 1,000 genetic isolates of a population of the microcrustacean *Daphnia pulex* demonstrates that temporal variation in selection intensity has a significant influence on nucleotide polymorphism and divergence. Most nucleotide sites experience fluctuating selection with mean selection coefficients near zero, with little covariance in selection strength across time intervals, and with selection distributed across large numbers of genomic islands of linked sites. These results raise challenges for the conventional interpretation of measures of nucleotide diversity and divergence as measures of random genetic drift and intensities of selection.

No biologist needs convincing that natural selection is a powerful force influencing essentially all organisms. Less clear, however, is the magnitude and temporal dynamics of selection operating across genomic regions and across time in natural populations. Hundreds of studies have focused on the measurement of fitness functions associated with quantitative traits, and many more have pursued genome-wide scans of divergent lineages in attempts to pinpoint nucleotide sites under purifying vs. positive selection (1–3). Whereas the first class of studies yields direct short-term measures at the level of complex traits, the targets of study are generally chosen because they are thought a priori to be under a particular form of selection, and the molecular underpinnings are generally left unstudied (4, 5). In contrast, whereas the second class of studies is focused at the molecular level, the time scale of divergence is commonly on the order of many thousands to millions of generations, and numerous unobserved aspects of demography and population structure can obscure the interpretation of patterns of variation (6). Recent studies of evolving laboratory populations have greatly enhanced our understanding of the origin of adaptations at the molecular level, but such studies generally focus on a specific induced selective challenge and/or initiate with a synthetic population structure, often relying on the emergence of de novo mutations from a single starting genotype (7–11), quite unlike the situation in natural populations.

Work with *Drosophila* populations has started to shed light on the degree of temporal stability of allele frequencies in natural settings. For example, studies on within-year changes in allele frequencies have revealed cyclical cycles (12, 13), as suggested earlier by Dobzhansky (14), although it is difficult to rule out a role for gene flow and spatial microheterogeneity in open populations of flies (15–18). A study of samples of *Drosophila melanogaster* separated by 35 y provides evidence for longer-term changes (19), although there are unresolved issues with respect to selection in very long-term laboratory cultures and on heterogeneity of field sampling sites. Small mesocosms seeded with replicate samples from a synthetic population provide further opportunities for studies of this sort, although the genetic architecture of the base population can be substantially altered with respect to that in the wild (20).

To help close this loop in our understanding of the operational features of natural selection, evolutionary biology could profit from the study of genome-wide selection in natural populations on time scales that minimize ambiguities in interpretations, and in structural settings that do not intentionally introduce novel selection pressures (21, 22). For metazoans and land plants, such investigations can require multiple years of sampling

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[1] To whom correspondence may be addressed. Email: mlynch11@asu.edu.

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and a substantial amount of genome sequencing. Here, we report on ten consecutive years (~35 generations) of population-genomic sequencing of a closed and undisturbed population of *Daphnia pulex,* a cyclically parthenogenetic microcrustacean that inhabits thousands of intermittent ponds across North America. Our results show that although selection is pervasive across the genome, the genome is also in near steady state with respect to selection, with average selection coefficients experienced by nucleotide sites near 0.0, but with substantial variance in selection coefficients across genomic sites and years, and with little temporal correlation in selection within sites. Many aspects of these results are qualitatively consistent with the concept of quasi-neutrality, wherein individual nucleotide sites experience random fluctuations in the direction and magnitude of selection, owing to shifting environmental conditions and/or stochastic changes in patterns of linkage disequilibrium (23, 24).

Results

Ten Sequential Years of Population-Genomic Data. This study utilizes a temporal series of genome sequence data from annual population samples consisting of 72 to 92 diploid individuals from a single temporary-pond population of *D. pulex,* from Portland Arch (PA), Indiana (*SI Appendix,* Text). Each sample was obtained during resting-egg hatch-out (typically mid-March to mid-April) and hence represents first-generation sexually produced offspring emerging prior to the operation of selective events during the subsequent ~3 to 5 generations of clonal reproduction. Horizontal hauls of a zooplankton net throughout the pond minimized potential effects of microheterogeneity by sampling across any possible microhabitats. Generally, the population enters a phase of sexual reproduction and resting-egg production by June, as the pond dries up and remains so until the following spring. As expected for a population lacking internal structure, genotype frequencies at the time of sampling closely adhere to Hardy–Weinberg expectations (25), as is true for most other temporary-pond populations of *D. pulex* (26).

After extraction of DNA, the clonal samples were barcoded, multiplexed, and sequenced to an average of 9× coverage per clone in the first 9 y (and to 38× in the final year) to generate 100 to 150-bp paired-end reads, which were then mapped to the high-quality reference assembly for a clone taken from an adjacent population (*SI Appendix,* Text). Following various filters for quality control (26; *SI Appendix,* Text), allele-frequency estimates were obtained with a maximum-likelihood procedure, which accounts for errors associated with sequencing and other nonbiological effects (27).

Rejecting the Hypothesis of Random Genetic Drift. Prior to analyses on the effects of selection on allele-frequency change, we verified that the magnitudes of allele-frequency fluctuations between annual samples are too large to be consistent with genetic drift associated with random allele sampling (*SI Appendix,* Text). Using a method-of-moments estimator that eliminates sampling error as a contributor to the variance of allele-frequency change, we obtained estimates of the effective population size (N_e) necessary to account for the standardized variance of allele-frequency change by drift alone. For the full range of temporal intervals, allele frequencies, and chromosomal locations, these analyses suggested that N_e would have to be no greater than 1,000 to 7,000 if the observed changes in allele frequency were solely a product of drift. These estimates are quite inconsistent with prior estimates of long-term N_e based on standing variation. Under the assumption of drift-mutation equilibrium, levels of silent-site diversity in the PA population imply $N_e \simeq 600,000$, and studies of nine other populations in the same geographic region and with similar ecologies have long-term N_e in the range of 430,000 to 750,000 (25, 26, 28).

To determine whether hatching of residual resting eggs from multiple years might bias the short-term estimates of N_e (an egg-bank effect), we performed computer simulations with various levels of retention of resting eggs from year to year, comparing the extracted estimates of N_e from the resultant series of allele frequencies with those obtained under the assumption of zero resting-egg retention (*SI Appendix,* Text). Egg-bank retention leads to a reduction in the perceived effective population size, as multiple generations of drift occur for older recruits. However, the effect is independent of the annual effective size and of the allele frequency, with a fairly small magnitude, <10 and <20% reduction with egg-retention rates as high as 30 and 50% (which seem highly unlikely).

Taken together, these results suggest that the observed temporal changes in allele frequencies reported on below are generally too large to be reasonably attributable to random genetic drift. This is not to say that all nucleotide sites are under direct selection, as the behavior of polymorphisms at essentially neutral sites will still be influenced by linkage disequilibrium with selected sites.

Mean and Temporal Variance of the Selection Intensity. Our goal is to generate a general understanding of the strength of selection operating at the nucleotide-site level across the entire genome. Given the time scale of the current study, we cannot expect to estimate with high accuracy the selection coefficient s at any particular site where the latter has an absolute value <0.01 (29). However, drawing from the $1,394,659$ informative sites used in this study (having frequency estimates across all sampling dates), it is possible to estimate the average intensity and variance of selection operating on sites of specific functional significance and in particular chromosomal locations. Nearly all polymorphic sites in the study population contain just two alleles (>97%; 26), but as we cannot yet be certain of the ancestral states at individual sites, the following analyses are oriented on minor alleles at biallelic sites (i.e., those with average frequencies across all years < 0.5); the estimated selection coefficients for major alleles have identical absolute values and simply differ in sign.

We start by considering the distribution of site-specific $\bar{s}$ (i.e., the 10-y average annual selection coefficient for each site) across the genome, restricting analyses to sites with adequate coverage and passing other quality filters shown by computer simulations to yield nearly unbiased estimates of s (*SI Appendix,* Text; 29). The distributions of site-specific $\bar{s}$ are quite symmetrical, with a mean of 0.00297 (SE = 0.00004) over all minor-allele frequency (MAF) classes (Fig. 1 *A* and *B*). Although there are significant differences between the mean $\bar{s}$ for different MAF classes, these fall in the range of 0.00030 to 0.00836 (all with SEs in the range of 0.00010 to 0.00012). Thus, on average, minor-alleles are under weak positive selection as a consequence of direct or (more likely) indirect effects associated with adjacent sites in linkage disequilibrium. Although the widths of the distributions decline with increasing MAF, this is largely a consequence of the sampling variance declining approximately inversely with increasing allele frequency (*SI Appendix,* Text).

Subtracting the average sampling variance of individual s estimates from the raw among-site variance of s within each year provides estimates of the true variance of s among sites, yielding average values in the range of 0.0047 to 0.0116 over different MAF classes (Fig. 1*B*). Despite the relatively large sample sizes in this study, these among-site variances constitute only ~7.8% of the sampling variance in s estimates among sites (within years).

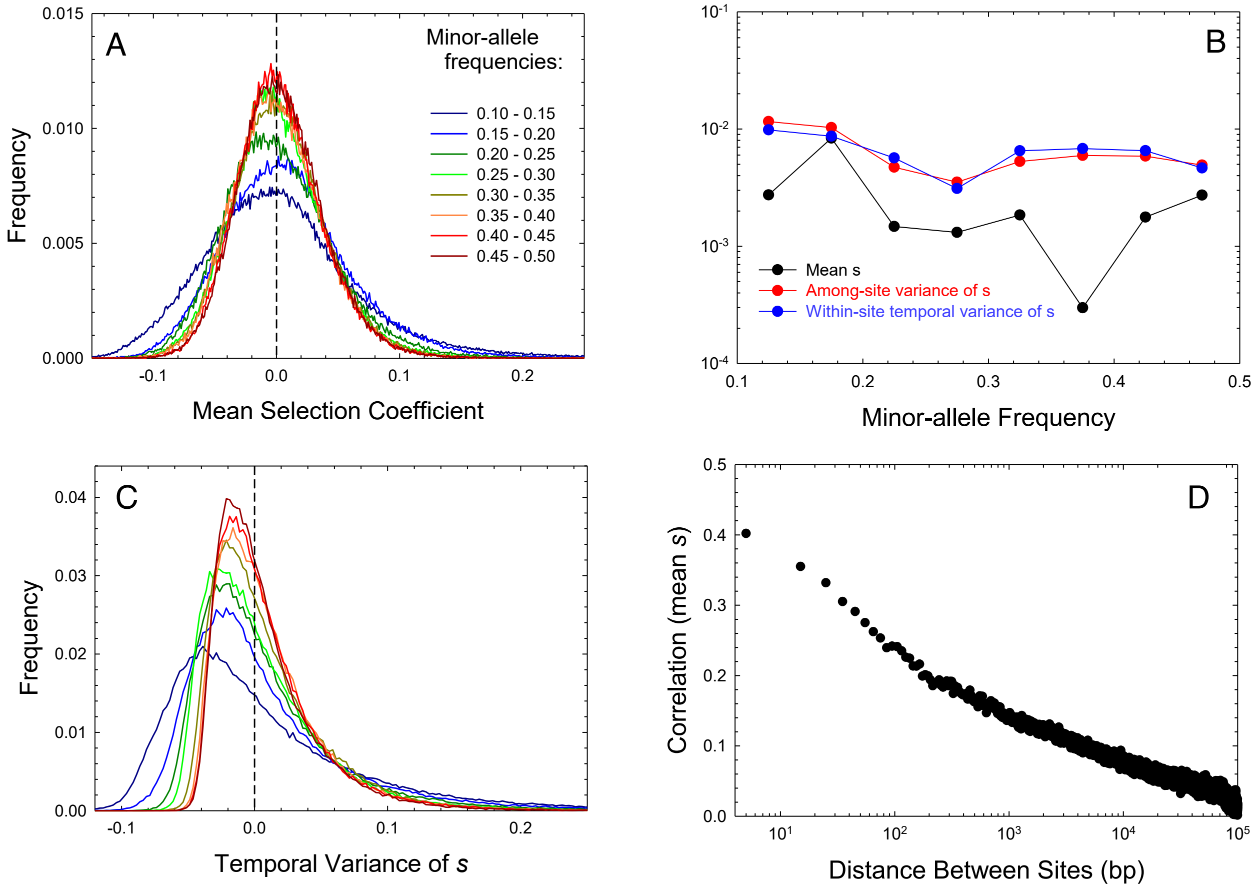

Fig. 1. Features of site-specific selection coefficients across the entire genome. (*A*) Distribution of time-averaged (mean) selection coefficients, $\bar{s}$, for the full set of genomic sites, given for different ranges of minor-allele frequencies (MAFs). (*B*) Means of site-specific $\bar{s}$ (black points), variance of $\bar{s}$ among sites after removal of sampling variance (red points), and the average temporal variance of site-specific *s*, again after removing variance associated with sampling (blue points), as a function of MAF. Note that in all cases the SE of the estimates are on the order of the width of the plotted points or smaller. (*C*) Distributions of the site-specific estimates of temporal variances of *s* (after removing the contribution from sampling error). (*D*) Correlations between $\bar{s}$ for pairs of sites separated by intrachromosomal distances of 0 to 10^5 bp, in increments of 10 bp.

The overall analyses suggest that in any particular year, individual genomic sites have net selection coefficients with ranges on the order of ±0.1 to ±0.2, although the range in $\bar{s}$ (mean site-specific s across generations) is much narrower (Fig. 1*A*).

By further using the nine single-year estimates of s for each site, it is possible to estimate the site-specific temporal variance of selection, σ_s^2, again after subtracting out the contribution from variance associated with sampling error. The sample distributions of σ_s^2 tend to be highly skewed, again with greater variance at lower MAFs resulting from elevated sampling error of the corrected estimates (Fig. 1*C*). Many estimates of σ_s^2 are negative, which is a necessary consequence of the use of an unbiased method-of-moments estimator with an expectation near zero. However, the average values of σ_s^2 across sites with different MAFs fall in the range of 0.0031 to 0.0098, with a mean value $\simeq 0.0065$ (Fig. 1*B*). These results are qualitatively similar to, although much more precise than, those obtained 35 y ago from temporal series of allozyme-allele frequencies in different *Daphnia* populations (30).

It again bears emphasizing that selection coefficients estimated in natural populations reflect the joint influence of any direct selection operating on the target site and of the aggregated indirect effects of all sites in linkage disequilibrium with the target site. Prior work with *D. pulex* has revealed the persistence of linkage disequilibrium out to 100-kb distances (25, 26, 28), suggesting that the net selection associated with particular sites is likely to be a function of the cumulative effects of linkage blocks tens of kb in length. To illustrate this point, we evaluated the correlation between $\bar{s}$ for sites separated by increasing physical distances on chromosomes. Such correlations decline monotonically with increasing physical distance, dropping by a factor of ten beyond a distance of 100 kb (Fig. 1*D*).

Presumably owing to the aggregated effects of linked sites, the differences in average magnitudes of short-term selection on sites with different functional significance are small. For example, for the subsets of zerofold redundant (amino-acid replacement) and fourfold redundant (silent) sites with negative s estimates, the average selection coefficients are −0.0320 vs. −0.0306, whereas for those estimated to be under positive selection, the average selection coefficients are 0.0373 vs. 0.0354 (SEs in all cases are < 0.00005). Averaging over the full distributions of selection coefficients, the minor alleles at 0-fold redundant sites are under slightly stronger positive selection, 0.00095 (SE = 0.00004), than are those at fourfold redundant sites, 0.00082 (0.00003).

To summarize to this point, the average minor allele is subject to a 10-y mean annual absolute selection coefficient of order 10^{-3} (with a biological range of approximately ±0.02 among sites), and a larger average among-year SD of s in the range of 0.06 to 0.10. Note that all of these selection coefficients are measured on an annual time scale, so with ∼4 generations per year, $\bar{s}$ should be reduced by this same factor to yield average per-generation estimates (assuming consistent selection across gener-

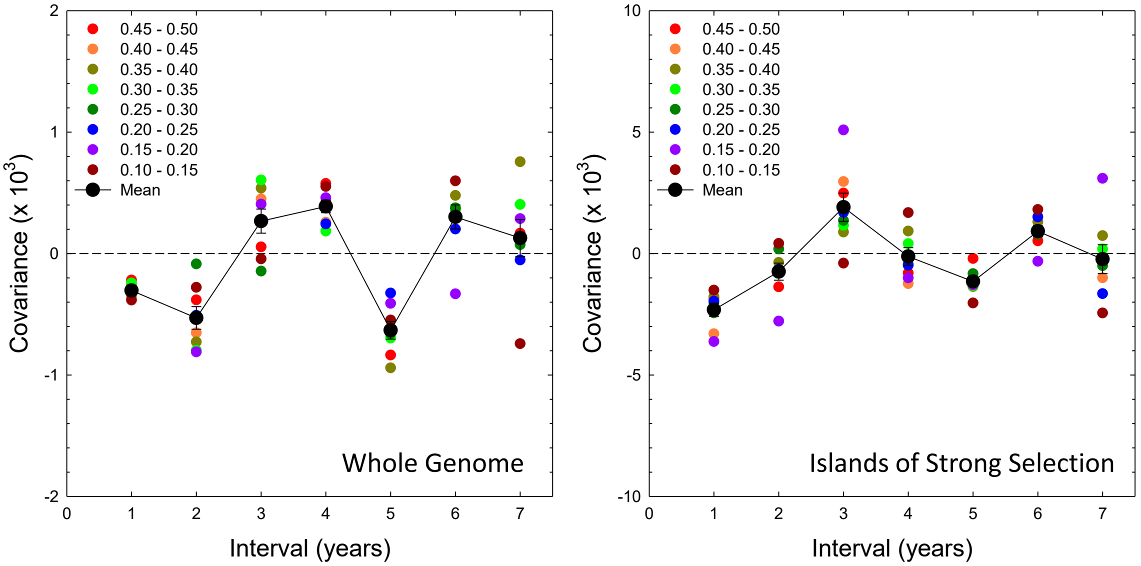

Fig. 2. Genome-wide temporal covariance of allele-frequency changes (standardized by the allele-frequency variance) across time intervals with increasing length. With ten consecutive annual sampling points, there are eight ways to compute single-year changes in adjacent years, seven ways to compare single-year changes separated by 2 y, etc., and the plotted values denote the means of such sets. Results are given for windows of MAFs and as averages for the full set of polymorphic sites (denoted in the *Inset*). The analysis on the left envelopes the entire genome, whereas that on the right is restricted to islands of strong selection (ISSs).

ations within years). In *SI Appendix*, Text, it is shown that the potential effects of a residual egg bank on these estimates are minimal for σ_s ($<10\%$ upward bias), and although more pronounced for $\bar{s}$ estimates, not enough to alter the conclusions noted above.

Temporal Covariance of Selection Intensity. If temporal series of s are independent, subtracting 1/9th of the average within-site temporal variance of s from the raw sampling variance of $\bar{s}$ should provide unbiased estimates of the true variance of $\bar{s}$, as this will factor out the variance of $\bar{s}$ associated with sampling error. However, on average, all such estimates are negative (in the range of -0.005 to -0.011 across MAF classes). Thus, temporal covariance in selection intensities must be occurring, and some of these covariances must be negative. In principle, selection coefficients at individual nucleotide sites can exhibit temporal correlations owing to persistent environmental fluctuations, linkage effects, and/or certain forms of balancing selection.

To quantify the degree to which annual selection coefficients associated with individual sites covary from year to year, we estimated the within-site covariance of s for intervals separated by $T = 1, 2, 3, 4,$ and 5 y, using unbiased estimators (*SI Appendix*, Text). A linear representation of the selection intensity operating on a site between adjacent time points i and $i + 1$ is

$$s_{i,i+1} = \bar{s} + \delta_i + e, \qquad \textbf{[1]}$$

where $\bar{s}$ is the mean selection coefficient over time (the subject of the previous section), δ_i is the deviation from the expectation in generation i associated with environmental and/or background genetic effects on selection, and e is the sampling error associated with estimation.

In the following analyses, $T = 1$ refers to paired selection coefficients separated by an entire annual interval, e.g., s based on years i and $i + 1$ vs. s based on years $i + 2$ and $i + 3$, and each time point enters an analysis no more than one time. As a consequence, the sampling errors (e) of the interval-specific s are entirely independent, so that the temporal covariance in selection coefficients for a particular site is equal to the covariance of selection deviations (δ) of observed interval-specific s from $\bar{s}$. That is, the within-site covariance of annual selection coefficients separated by intervals of T years is an estimate of $\sigma(\delta_0, \delta_{1+T})$. As noted in *SI Appendix*, Text, for each T, between one and three configurations of samples fulfill the requirement of independence of samples in the estimation of temporal covariances of s.

Although such analyses were performed separately for each MAF interval, there was no significant gradient in covariances with respect to MAFs. Averaging over sites, temporal selection covariance is significantly negative for intervals of $T = 1$ and 4 [-0.00258 (SE $= 0.00054$) and -0.00468 (0.00101), respectively], significantly positive for intervals of $T = 3$ and 5 [0.00249 (0.00065) and 0.00199 (0.00078), respectively], and nonsignificant for $T = 2$ (-0.00023 (0.00118)). The degree to which such covariances are a consequence of interannual changes in ecology, shifting weather patterns, etc., is unclear, and the patterns observed should not be construed as general cycles involving such factors. Rather, they are likely to be serendipitous outcomes of events in the short 10-y interval of this study.

The primary point is that, on average, some temporal covariance in selection does exist for individual sites. However, the overall absolute values of these covariances are all smaller than the estimated average temporal variances, meaning that the temporal correlations are not large. In addition, there is no gradual reduction in the temporal covariance of s with time, which is inconsistent with a progressive dissipation of correlations between environmental factors over time. Finally, it should be noted that a negative selection covariance does not necessarily imply a change in the sign of s across generations, as such patterns can also arise when the sign of s remains the same but with more extreme values of s in one interval being followed by less extreme values in the next.

Genome-Wide Temporal Covariance of Selection. To gain further insight into the extent to which this population is experiencing consistent forms of selection across time, we also applied the method of Buffalo and Coop (31) to estimate the

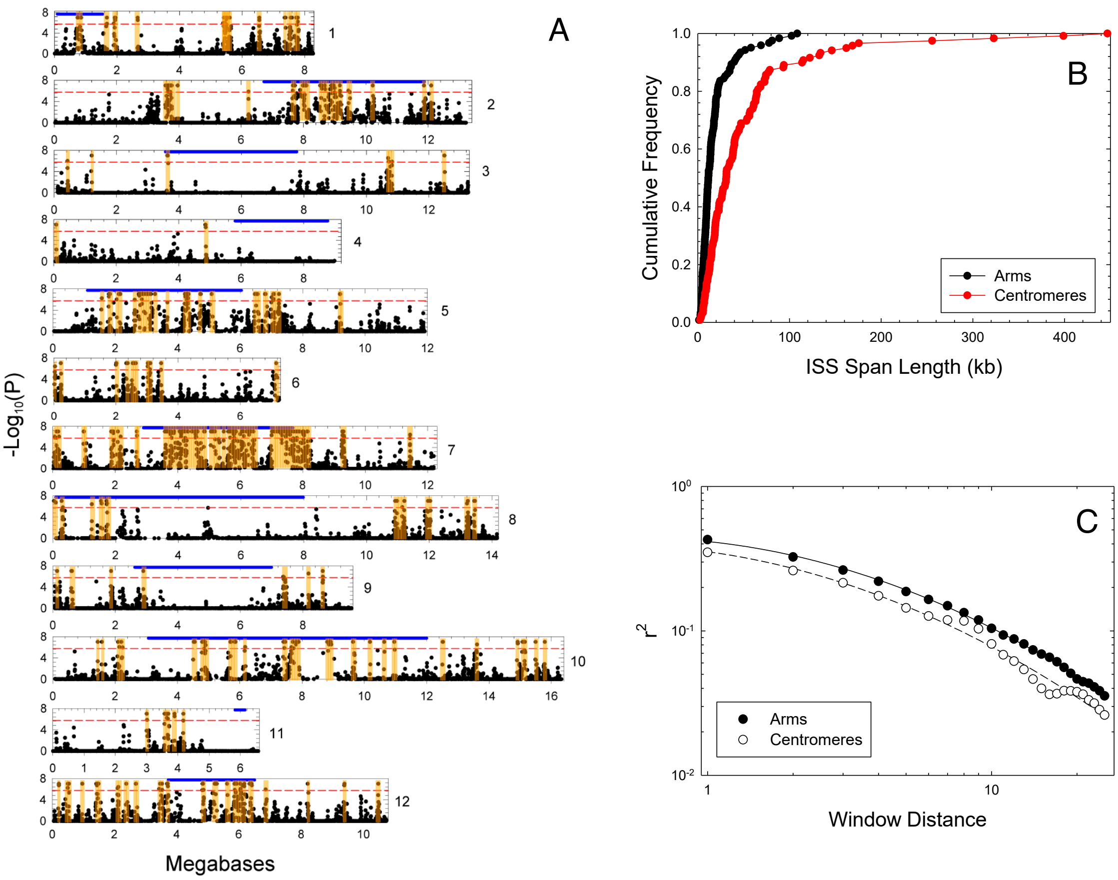

Fig. 3. Chromosome-wide scans of window-specific intensities of selection. (*A*) For the set of SNPs within each window, the average estimate of absolute values of selection coefficients was compared with the probability distribution based on samples of randomized sites, with *P* denoting the probability of achieving the observed measure by chance. The dashed red lines denote the critical cutoff points for significance after correcting for multiple comparisons. Horizontal blue bars denote the approximate locations of centromeric regions based on multiple laboratory crosses (33). Yellow vertical bars denoting the positions of ISSs are often slightly wider than actual ISS spans (for visualization purposes). (*B*) Cumulative frequency distributions for the lengths of ISS blocks residing on chromosome arms vs. windows. (*C*) Decline of the correlation of window-specific selection strengths with increasing distance, where $x = 1$ denotes adjacent (nonoverlapping) windows, and the curves are fitted quadratic regressions.

genome-wide covariance of allele-frequency changes across time intervals (see also ref. 18). The approach here is complementary to that in the preceding section, but rather than focusing on individual nucleotide sites, the goal is to evaluate whether the changes in frequencies for the full set of polymorphic sites are correlated across two sets of time intervals, as might be expected for a polygenic trait experiencing directional selection. With allele-frequency changes available for nine different single-year time intervals, 36 pairwise comparisons could be made, ranging from eight involving single-year intervals to one separated by 8 y. Using the approaches advocated by Buffalo and Coop (32), we performed such analyses for the pooled set of all genomic sites as well as for those in narrower MAF windows, in each case standardizing the covariance measures by dividing by $p(1-p)$ to account for the expectation that the magnitude of selection is proportional to the heterozygosity (*SI Appendix*, Text).

The majority of such standardized covariance estimates are very close to zero (Fig. 2). Nonetheless, for time intervals of 1, 2, and 5 y, estimates for the pooled set of sites are significantly less than zero, and the estimates for all MAF classes are negative as well. In contrast, for intervals of 3, 4, and 6 y, the average standardized covariances of allele-frequency changes are significantly positive. Although not significantly different from zero, the estimates for intervals of 7 and 8 y are quite noisy, as these are based on just two and one comparisons, respectively. The overall conclusion here is that the study population reveals no evidence of consistent directional selection at the polygenic level, with the negative covariances for several intervals being potentially consistent with some form of fluctuating stabilizing selection.

Chromosome-Wide Distributions of Selection Intensity. To further evaluate the degree to which the average strength of selection associated with nucleotide sites is an indirect function of that operating on linked sites, we estimated the average absolute value of $\bar{s}$ over all sites within blocks of 100 consecutive sites with MAFs > 0.1, proceeding along the full lengths of chromosomes, with adjacent blocks overlapping by 50%. We also drew 10^7 random sets of such SNPs from the entire genomic pool of data to calculate the expected distribution of average s in the absence of linkage effects. By comparing the observed ($|\bar{s}| - 2$ SEs) for each window (i.e., making the estimate less extreme) with the cumulative random probability distribution, we were then able to determine the conservative probability P of obtaining a window-specific $\bar{s}$ more extreme than that observed. The overall survey evaluated the features of 27,876 windows with average and SD of length 9.28 (SE $= 0.09$) and 14.95 kb. Such windows typically contain 0 to 3 protein-coding genes, as the average gene span in *D. pulex* is ~10 kb. After correcting for multiple comparisons, we used a critical P value of 1.80×10^{-6} as a cutoff for windows with

Table 1. Estimates of within-population diversity measures (π) for synonymous (S) and nonsynonymous (N) sites in protein-coding genes, as well as for divergence (d) statistics from comparisons with orthologous genes in the outgroup species *Daphnia obtusa*

Region	Mb	Genes	Pop.		π_N	π_S	π_N/π_S	d_N	d_S	d_N/d_S	NI
ISS arms	2.2	267	0.66	Mean	0.0029	0.0159	0.2136	0.0243	0.1448	0.1660	1.588
				SE	0.0000	0.0000	0.0012	0.0002	0.0003	0.0008	0.008
				Median	0.0016	0.0135	0.1581	0.0189	0.1383	0.1343	1.110
ISS cent.	6.1	355	0.49	Mean	0.0023	0.0090	0.3797	0.0304	0.1368	0.2082	2.264
				SE	0.0000	0.0000	0.0027	0.0003	0.0005	0.0014	0.011
				Median	0.0015	0.0061	0.2457	0.0160	0.1192	0.1281	1.600
Non-ISS arms	76.8	10,848	0.79	Mean	0.0029	0.0198	0.2000	0.0236	0.1401	0.1528	1.476
				SE	0.0000	0.0000	0.0002	0.0000	0.0000	0.0001	0.001
				Median	0.0017	0.0091	0.1978	0.0143	0.1236	0.1129	1.390
Non-ISS cent.	43.8	3,480	0.59	Mean	0.0024	0.0135	0.2712	0.0246	0.1369	0.1781	1.868
				SE	0.0000	0.0000	0.0002	0.0000	0.0000	0.0001	0.001
				Median	0.0015	0.0106	0.1603	0.0153	0.1257	0.1169	1.370

Total length of regions is in units of Mb; Genes refers to the total number of genes analyzed; Pop. refers to the fraction of genes with uniquely mapped reads from the population surveys; NI denotes the mean neutrality index (ratio of π_N/π_S to d_N/d_S). Data are given separately for chromosomal arms vs. centromeres, and for genes in islands of significant selection (ISSs) vs. non-ISSs. All statistics are averages over all genes, with SEM given below the means.

$\bar{s}$ significantly elevated over the random expectation. This analysis revealed both large linkage blocks and punctate regions of one or more adjacent windows exhibiting significantly strong collective selective effects (Fig. 3*A*). Regions of significant selection are found on nearly every chromosome arm and centromere.

To gain further insight into the spatial patterning of selection and its consequences, we delineated ISSs as chromosomal blocks harboring adjacent windows with high significance, with each span constructed by starting with windows deemed individually significant and then extending to adjacent segments with $P < 10^{-4}$. This treatment with a somewhat reduced P level in adjacent windows is necessary because: 1) the breakpoints associated with islands of interest will often be embedded within end windows, thereby diminishing their overall probability levels; and 2) such a "borderline" window may be immediately followed by another string of significant windows and so may be a "false negative" (even though it is still significant at this lower probability level). The approach used here is quite conservative, as the multiple-comparison correction conditional on a small number of local windows is much less stringent than that for the entire genome.

This sliding-window analysis led to the identification of 241 ISSs, 122 of which reside on chromosome arms, ranging from 1.4 to 108.5 kb in length. The average number of ISSs per Mb within centromeric regions is 1.7× greater than that on chromosome arms, 2.5 (SE $= 0.7$) vs. 1.5 (0.2), and the average ISS span within centromeric regions is nearly 3× that for ISSs on chromosome arms: 51.7 (SE $= 6.3$) vs. 18.3 (1.8) kb (Fig. 3*B*). As a result, a greater fraction of centromeric regions is occupied by ISSs than is the case for chromosome arms, 0.125 (0.025) vs. 0.028 (0.004). This may in part be a consequence of the lower levels of recombination in centromeric regions, and the resultant effects of linked selection. However, although little crossing-over is revealed in centromeric regions in single-generation laboratory crosses, the long-term average rate of recombination is only suppressed ∼2-fold relative to the average rate on chromosome arms (28), and the decline in the correlation between window-specific mean s estimates is similar in both types of chromosomal regions (Fig. 3*C*). Restricting the analysis in the preceding section to sites within ISSs confirms the genome-wide temporal patterns, but yields estimates that are ∼5× greater in absolute value (Fig. 2). Given that <10% of the genome is associated with such regions, these observations are consistent with a high degree of enrichment of strongly and cohesively selected sites within these regions.

Polymorphism and Divergence of Potential Targets of Selection. There are several unique features of the genes contained within ISSs (Table 1). First, ∼42% of them are young gene duplicates (<2% sequence divergence between paralogs), in contrast to ∼ 31% of such genes in non-ISS regions. A large fraction (0.46) of these ISS duplicates have no obvious orthologs in other metazoans and may be *Daphnia*-specific genes. (To search for orthologs, we subjected each annotated *D. pulex* gene to Blastp analysis against the nonredundant NCBI protein-coding database, regarding any gene with <30% sequence coverage (with E-value >0.00001) for all non-*Daphnia* species as potentially unique to *Daphnia*). Second, ISS genes exhibit significantly lower levels of average silent-site diversity (π_S) but nearly the same levels of replacement-site diversity (π_N) as non-ISS genes, with the π_N/π_S ratio in the former being 1.1× (arms) to 1.4× (centromeres) higher than in the latter. Third, with respect to average divergence from the closely related outgroup species *D. obtusa,* the ISS genes exhibit higher levels of d_N and d_N/d_S, the latter being 1.1× (arms) to 1.2× (centromeres) higher than that in non-ISS regions, whereas the estimates of d_S are nearly identical. As a consequence of the greater elevation of π_N/π_S relative to d_N/d_S, the neutrality index, NI = $(\pi_N/\pi_S)/(d_N/d_S)$ (with NI $= 1$ implying neutrality, on average) is 10 to 20% greater in ISS than in non-ISS genes. As levels of polymorphism and divergence are cumulative products of evolutionary factors operating over long time scales, the existence of such distinguishing features of ISSs suggests that the regions involved are not simply fortuitous responders to the unique environmental conditions encountered during the limited 9-y span of this study, but must at least partially reflect longer-term consequences of ecological and/or linkage-disequilibrium issues.

The preceding observations are qualitatively consistent with predictions from theoretical population genetics, while also motivating the need for further work in this area. For example, for the special case in which mutation is unbiased (with rate u per nucleotide site) and $\bar{s} = 0$, the steady-state distribution of allele frequency x with drift, reversible mutation, and fluctuating selection (with temporal variance σ_s^2) is given by

$$\phi(x) = \frac{C \cdot [x(1-x)]^{\theta-1}}{[1+\gamma x(1-x)]^{\theta}}, \qquad [2]$$

where $\theta = 4N_e u$, $\gamma = 4N_e\sigma_s^2$, and C is a normalization constant such that the integration of Eq. 2 over the full allele-frequency spectrum is equal to 1.0 (34, 35). Multiplying $\phi(x)$ by $2x(1-x)$ and integrating over $x = 0$ to 1 yields an estimate of the expected nucleotide diversity (π) under fluctuating selection.

The average level of silent-site diversity across the genome in the study population is 0.014, and under the assumptions of neutrality, this kind of measure is traditionally interpreted as being an estimate of θ, which after factoring out the known mutation rate (36) yields $N_e \simeq 600{,}000$ for the study population. If this conventional interpretation of π_s is close to correct, the above estimates of σ_s^2 imply that γ may often be on the order of 10^4 or even higher. How might such variation in selection intensity influence standing levels of diversity? From Eq. 2, it can be seen that if $0 < \gamma < 10$, the denominator will generally be close to 1 (assuming $\theta \simeq 0.014$), so that $\phi(x)$ will be closely approximated by the numerator alone, which is equivalent to the neutral expectation. Solution of Eq. 2 shows that for $\gamma = 10^3$, 10^5, and 10^7, expected levels of heterozygosity relative to θ decline to 0.94, 0.89, and 0.84, respectively.

Although this analysis suggests that fluctuating selection causes standing levels of silent-site diversity to underestimate θ (and hence the N_e estimate) in the study population by as much as 15%, the actual depression is likely greater. One issue with Eq. 2 is that it presupposes some extrinsic measure of N_e in the definition of γ, whereas the latter is actually defined in part by fluctuations in selection, i.e., by σ_s^2 itself. This can be shown with computer simulations in a Wright–Fisher framework with consecutive episodes of reversible mutation, selection (assuming a Gaussian distribution of s), and drift (*SI Appendix*, Text). By following the behavior of a selected site and a completely linked neutral site (mutating independently at the same rate), the long-term mean values of π_S and π_N can be evaluated.

In the absence of selective interference, the mean nucleotide diversity at the silent site is $4Nu/(1 + 4Nu)$, where N is the effective population size in the absence of selection. However, the simulation results show that with increasing N, mean silent-site diversity begins to decline once N exceeds $\sim$1,000/σ_s^2, with a 100-fold increase in N beyond this point leading to a nearly $\sim$ 100-fold decline in π_s (Fig. 4*A*). Below this critical point, the behavior of diversity at the linked neutral and selected sites remains equivalent, so that $\pi_N/\pi_S = 1$, but with increasing N, π_N/π_S begins to exceed 1, and with large (but realistic) values of σ_s^2, π_N/π_S can exceed 10 (Fig. 4*B*). In this domain, π_N is depressed below the neutral expectation of $4Nu$, as anticipated by Eq. 2, but still grows slowly with increasing N while π_S is declining. Although nonzero $\bar{s}$ results in lower π_N/π_S, the tendency for π_N/π_S to exceed 1.0 still occurs with large $N\sigma_s^2$. This type of behavior results because while fluctuating selection tends to encourage the maintenance of variation at selected sites by imposing a sort of frequency-dependent selection, it also magnifies the fluctuations in allele frequencies, which reduces the depth of gene genealogies, thereby reducing the expected value of π_S (37, 38).

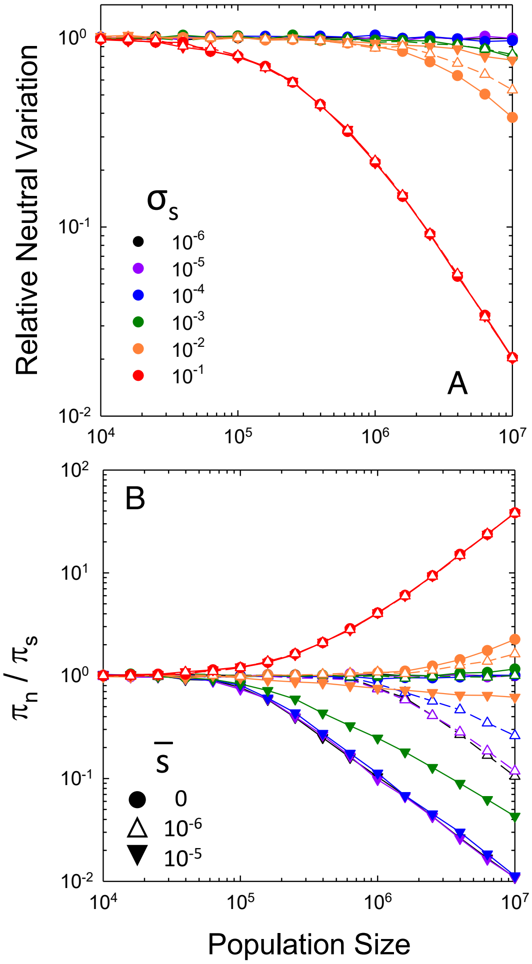

Fig. 4. Response of expected within-population measures of nucleotide diversity as a function of the temporal SD of the selection coefficient (σ_S) and the population size (N). (*A*) Nucleotide diversity at a linked silent site (π_S) relative to the neutral expectation at drift-mutation equilibrium under free recombination. (*B*) Ratio of diversity at the selected site to that at a completely linked neutral site, π_N/π_S. Color legend in the upper panel designates various levels of the temporal SD of the selection coefficient s; *Inset* in the *Lower* panel designates the symbol shapes used for three levels of the average selection coefficient $\bar{s}$.

These predictions from theory are concordant with the observations on nucleotide diversity in Table 1—a decline in π_S, relative stability of π_N, and an increase in the ratio of the two in ISS vs. non-ISS regions. Theory also predicts that fixation probabilities will increase with fluctuating selection (23, 34, 39–41), which is consistent with our observations on d_N. There has been some suggestion that fluctuating selection will decrease the ratio of polymorphism to divergence, thereby leading to the false impression of positive selection (42). However, the latter work does not disentangle the effects of fluctuating selection on N_e, and the predicted pattern is not apparent in the data herein.

Discussion

Theoreticians have long appreciated the potential effects of fluctuating selection on patterns of nucleotide diversity and divergence (e.g., ref. 43; and references cited above), although in the absence of data on the magnitude of the variance in selection intensity (σ_s^2), most empirical studies in molecular population genetics implicitly assume that fluctuating selection is of negligible significance. This is a concern because the movement of allele frequencies by both random genetic drift and selection is proportional to the heterozygosity at a nucleotide site, and for the special case of $\bar{s} = 0$, the influence of fluctuating selection can be nearly indistinguishable from the effects of drift associated with gamete sampling (44). Despite this conceptual problem, hundreds of studies have interpreted patterns of population-genetic variation in terms of drift, mutation, and deterministic selection. For example, such a strategy remains central to virtually all studies using measures of nucleotide variation/covariation at putatively neutral sites to estimate N_e after factoring out known estimates of mutation and/or recombination rates. It is also central to studies relying on estimates of π_N, π_S, d_N, and d_S, and their various ratios to derive inferences about the relative strength of positive and purifying selection within and among genes and species.

The current study presents one of the first genome-wide analyses of temporal variation in selection in an isolated and unmanipulated natural population, and in doing so illustrates the significant ways in which this underappreciated issue can influence the interpretation of parameter estimates in molecular population genetics. Shorter-term studies of temporal variation in populations of the fruit fly *D. melanogaster* have been pursued, although there are uncertainties with respect to influences from gene flow, microhabitat heterogeneity, and the reliance on artificially constructed base populations (12, 13, 18). Our results show that net selection coefficients operating at the nucleotide-site level in *D. pulex* (on an annual time scale) are quite small in terms of absolute values, with the average selection coefficient operating on minor alleles often being in the range of 10^{-3} to 10^{-2} or smaller. For fourfold redundant sites, which are most likely to be functionally neutral, average estimates of $\bar{s}$ are closer to but still significantly different from 0.0 on average, presumably reflecting the effects of linkage disequilibrium with functionally relevant sites. Nevertheless, given the long-term effective population size of *D. pulex,* selection coefficients as small as 10^{-4} represent strengths of selection well beyond the power of random genetic drift.

Although we report on the results of just a single population in a single species, there is no evidence that the results are peculiar consequences of the reproductive life cycle of *Daphnia*. For example, despite the brief bouts of asexuality each generation, the population-genetic features of *D. pulex* are quite similar to those of the well-studied *D. melanogaster,* which actually has significantly less per-generation recombination per nucleotide site than the study population, owing to the lack of recombination in males and the substantially reduced number of chromosomes (26, 45). Nor does the possible existence of a resting-egg bank alter our results in a substantive way (*SI Appendix*, Text).

Notably, in analyses of the genome-wide temporal covariance of allele-frequency change in a natural population of *D. melanogaster,* using data from Bergland et al. (12), Buffalo and Coop (32) and Bertran (18) found a similar pattern to that reported in Fig. 2—on average, weak but negative covariance over the shortest interval and near-zero covariance thereafter. Likewise, although the methods employed were quite different, a 10-y genomic survey of frequency changes for common alleles in the monkey flower (*Mimulus guttatus*) revealed substantial fluctuating selection associated with numerous islands of linked selection and modest temporal covariance of s (21). Thus, observations from at least three species are consistent with the hypothesis of significant fluctuations in selection intensities operating at the level of nucleotide sites, often with very little autocorrelation in selection across years.

Although it has been argued that significant genome-wide covariance of allele-frequency change is an indicator of pervasive selection on polygenic traits (31, 32), in the absence of information on the identity of selected sites, the meaning of such measures remains unclear. The resultant measures are likely greatly diluted by the behavior of unselected sites as well as by mixtures of sites experiencing positive vs. negative temporal covariance of selection. As a consequence of the latter, an absence of genome-wide covariance of allele-frequency change need not imply an absence of selection. These points are illustrated by the substantial inflation of estimated covariances of allele-frequency changes when analyses are restricted to ISSs (Fig. 2). It is also worth emphasizing that negative covariances need not indicate sign changes in selection across intervals, and it is theoretically possible for positive covariances to arise in the face of sign changes. To bridge the gap between molecular population genetics and quantitative genetics, future studies of this nature will be more revealing if they can be focused on specific quantitative traits and their known constituent loci, admittedly a daunting task for any species.

Despite the central role of stabilizing fitness functions in the foundations of quantitative-genetic theory for natural populations, there has been growing skepticism that selection on polygenic traits is typically stabilizing in nature at any specific point in time (46–51), as well as an increased appreciation for the effects of fluctuating selection on quantitative-trait loci (38, 52). In principle, temporally shifting selection pressures can lead to the illusion of long-term stabilizing selection, although there is disagreement on the magnitude and meaning of variation in changes in selection gradients inferred in previous studies on the behavior of quantitative traits (47, 53–57).

Despite the lack of a direct link to specific quantitative traits (other than fitness), our results appear to be qualitatively compatible with the scenario of quasi-neutrality envisioned by Wright (56) and Kimura (23), whereby allelic variants have temporal average selection coefficients close to zero, while experiencing significant random variation in selection pressures across generations. Although there is some temporal covariance of selection experienced by individual nucleotide sites in *D. pulex,* this is on average quite small relative to the temporal variance of s, and might be revealed to be even closer to zero with a longer temporal series of data, rendering the overall temporal pattern of selection close to the idealized model of Wright and Kimura. Nonetheless, there is a compelling need for additional empirical work with different kinds of organisms to determine whether general phylogenetic patterns exist in genome-wide distributions of key composite parameters such as $N_e\sigma_s^2$.

What seems clear, however, is that rather than being entirely a consequence of simple stochastic effects of direct selection operating on individual nucleotide sites in isolation (as assumed in prior models), the observed features of each site are collectively driven to a large extent by the influences of selection operating on neighboring sites under linkage disequilibrium. Linkage blocks of sites under significant selection over the 10-y sampling period were often on the scale of 50 kb or smaller, although a few spans extend up to >150 kb in centromeric regions. Such observations are consistent with prior work in *D. pulex,* including in the study population, indicating that significant linkage disequilibrium is quite strong at distances $< 10^3$ bp and then gradually dissipates out to ~250 kb (25, 28).

Given that $N_e\sigma_s^2 = 1$ is the approximate benchmark above which fluctuating selection begins to play a role in determining patterns of molecular evolution, our observation that σ_s^2 is commonly orders of magnitude greater than the power of random genetic drift, $1/(2N_e)$ suggests that such fluctuating selection may play just as substantial a role as the effects of finite population size in determining the fates of mutant alleles. Of particular concern is the demonstration that increasing levels of fluctuating selection can differentially influence π_S and π_N in ways that can lead to the false impression of relaxed selection (e.g., π_N/π_S approaching 1.0) or strong balancing selection ($\pi_N/\pi_S > 1.0$). These observations motivate the need for further theoretical investigation into the extent to which fluctuating selection alters the ways in which patterns of molecular variation within and between species should be interpreted. The challenges are considerable, given that most existing theory treats N_e as a fixed parameter, ignoring the influence of σ_s^2 on N_e and the additional complications associated with nonzero $\bar{s}$ and non-Gaussian distributions of s. Because virtually all population-genetic theory in this area focuses on single loci, there is a need for the development of models that incorporate the effects of linked sites jointly influenced by fluctuating selection. No nucleotide site can be permanently immune to such effects.

Our results are also relevant to the common use of "Manhattan plots" based on chromosome-wide scans comparing isolated populations and/or species to identify candidate regions experiencing strong positive selection. The ISSs observed in this study are the outcomes of fewer than 40 generations of evolution. Thus, depending on the form of data analysis, this raises the possibility that many metapopulation studies may not be identifying long-term targets of local adaptation, but simply identifying the serendipitous outcomes of the most recent generations experienced by the populations involved. In the future, it will be useful to evaluate whether chromosomal regions that are most subject to selection within a population over short time scales (as observed here) are correlated with islands of divergence among subpopulations.

Finally, although science increasingly relies on rapid experimental analyses, nature proceeds at its own pace. Our results suggest that multigenerational population-genomic surveys, combined with ecological observations, will be essential to understanding the population-genetic processes and adaptations that are associated with stable and/or changing environments. Indeed, substantially longer surveys than that reported on here will likely be required to determine whether and why the chromosomal targets of selection shift over ecologically relevant time scales. Given the current support of long-term ecological observatory networks (e.g., NEON in the United States) and the much more substantial investment in understanding the history of the universe (e.g., the Webb telescope), the additional costs necessary for gaining insights into key microevolutionary processes in natural populations are not unreasonable. *Daphnia* populations inhabiting large permanent lakes offer considerable opportunities here, as their resting eggs can remain viably preserved in undisturbed sediments for up to 200 y. In principle, with enough sampling from sediment cores with stable stratigraphies (ideally, many dozens of genetic isolates per time horizon), population-level studies can be extended over time periods exceeding several hundreds of generations (57–61).

Materials and Methods

This study relies on a temporal series of ten samples from an isolated temporary pond population of *D. pulex,* derived from newly arisen annual cohorts of resting-egg hatchlings, in the Portland Arch Nature Conservancy. Full genomic sequences were obtained for ~90 individuals from each sample, using methods more fully described in *SI Appendix*, Text but following the same protocols as in prior work with this and other related populations (e.g., refs. 26 and 45). Methods for estimating selection coefficients and their sampling variance are derived from Lynch (30) and Lynch and Ho (29), using modifications outlined in *SI Appendix*, Text to reduce sampling bias and minimize sampling error.

Data, Materials, and Software Availability. Computer programs and raw sequence data have been deposited in Lynchlab GitHub and NCBI (NCBI PRJNA684968).

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Author affiliations: [a]Biodesign Center for Mechanisms of Evolution, Arizona State University, Tempe, AZ 85287; [b]Hubei Key Laboratory of Genetic Regulation and Integrative Biology, School of Life Sciences, Central China Normal University, Wuhan 430079, China; and [c]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

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