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THE DYNAMICS OF DIVERSIFICATION IN EVOLVING *PSEUDOMONAS* POPULATIONS

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Abstract.—Determining the mechanisms that promote the evolution of diversity is a central problem in evolutionary biology. Previous studies have demonstrated that diversification occurs in complex environments and that genotypes specialized on alternative resources can be maintained over short time scales. Here, we describe a selection experiment that has tracked the dynamics of adaptive diversification within selection lines of the asexual bacteria *Pseudomonas fluorescens* over about 900 generations. We cultured experimental populations from the same two isogenic ancestral strains in simple, single-substrate environments or in complex, four-substrate environments. Following selection we assayed the growth of genotypes from each population on each substrate individually. We estimated mutational heritability, V_m/V_E, as 1×10^{-3} per generation in simple environments and 3×10^{-3} per generation in complex environments. These values are roughly consistent with estimates reported in other systems. Populations selected in complex environments evolved into genetically diverse communities. Genotypes exhibited greater metabolic differentiation from other genotypes in their own population than to genotypes evolving in other populations, presumably as a result of resource competition. In populations selected in simple environments, little genetic diversity evolved, and genotypes shared very similar phenotypes. Our findings suggest that ecological opportunity provided by environmental complexity plays a major role in the evolution and maintenance of diversity.

Key words.—Adaptation, diversity, ecological opportunity, environmental complexity, experimental evolution, genotype-by-environment interaction, mutational heritability.

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If a lineage is introduced to a heterogeneous environment, it will be provided with ecological opportunity in the form of underexploited or novel resources. Divergent natural selection can then arise from differences in the resources among habitats and competition for shared resources within the same habitat (Schluter 2000; MacLean et al. 2005). Environmental heterogeneity has been implicated as a primary mechanism governing the origin of diversity since at least the Modern Synthesis (Fisher 1930; Muller 1942; Dobzhansky 1951). Until recently, however, there has been relatively little empirical work on the subject (Schluter 1996, 2000, 2001; Rundle and Schluter 2004; Rundle and Nosil 2005). Recent advances in the study of adaptive diversification have been made through better estimates of changes in phenotypes and resource use through time (Schluter 2000). The number of cases is small, however, and most of the evidence has been obtained by comparative methods rather than by experiments (but see Buckling et al. 2003; Kassen et al. 2004). Experimental studies are illuminating because they can document the historical selection pressures that have led to divergence (Schluter 2000). In particular, microbial microcosms are now recognized as having several advantages over performing field experiments (Jessup et al. 2004; Kassen and Rainey 2004). Short generation times and large population sizes, along with the relative ease with which both organisms and environments can be manipulated, make these systems very amenable to testing ecological questions over evolutionary timescales.

The simplest type of selection experiment involves introducing a single genotype of an asexual microbe to an environment containing just a single niche to which it is initially not well adapted. As the population grows, random mutations will lead to genetic diversification. A very small fraction of these mutations will be beneficial, in the sense that they confer a higher fitness than the ancestor. The fittest genotype among these mutants should eventually replace all others, leading to a genetically uniform population. This is the process of periodic selection, whereby evolution is characterized by a series of clonal replacements, and diversity is purged each time a beneficial mutant arises and sweeps through the population (Novick and Szilard 1950; Atwood et al. 1951).

If the environment is not uniform, but instead offers several distinct niches that differ in their conditions for growth, then this ecological opportunity can allow the evolution of a diverse community of genotypes (Geritz et al. 1998; Day 2000; de Meeus and Goudet 2000). Rainey and Travisano (1998) provided a critical test of this prediction by selecting a single clone of the bacterium *Pseudomonas fluorescens* in microcosms incubated either with or without spatial heterogeneity. In spatially heterogeneous microcosms the ancestral genotype diversifies into several genetically differentiated variants. In contrast, no diversity evolves in the spatially homogeneous microcosms, presumably because of the lack of ecological opportunity provided by distinct niches. This model system has been extensively used to test questions about the ecology and genetics of diversification (Travisano and Rainey 2000; Buckling and Rainey 2002; Buckling et al. 2003; Spiers et al. 2003; Brockhurst et al. 2004; MacLean et al. 2004, 2005). However, none of these studies has yielded insights into the dynamics of diversification on time scales longer than 100 generations.

Pseudomonas is one of the most diverse and ecologically significant groups of bacteria (Spiers et al. 2000). Members of the genus are abundant in all of the major natural environments (terrestrial, freshwater, and marine) and also form intimate associations with plants and animals (Spiers et al. 2000). This distribution suggests a remarkable degree of adaptability. Here we use *P. fluorescens* to examine the de novo evolution of diversity in media of differing ecological

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opportunity. Ecological opportunity is determined by environmental complexity, which can be thought of as a specific type of environmental heterogeneity that occurs when a spatially homogeneous environment contains several resources. In a previous experiment, we examined the role of environmental complexity in the evolution of niche breadth by selecting replicate lines of *P. fluorescens* in environments that varied in the number of available substrates (Barrett et al. 2005). This experiment showed that evolution in complex environments results in a genetically diverse population of overlapping generalists, each of which is adapted to a certain range of substrates but not all. Because populations were only sampled once, at the end of approximately 900 generations of selection, the experiment did not provide any information about the dynamics of diversification within each population. It was not possible, for example, to determine whether the evolved diversity accumulated linearly or nonlinearly through time.

In this paper, we describe an experiment that provides a temporal perspective on evolution in complex environments. A great advantage of experimental evolution with microbes is that it permits ''time-travel'' through the evolutionary history of a population. Bacterial cultures can be frozen at regular intervals throughout a selection experiment and revived for future analysis (Lenski et al. 1998). We selected replicate lines of *P. fluorescens* in five environments containing either one or four carbon substrates for about 900 generations. Approximately every 100 generations we froze a sample of each line, and at the end of the selection experiment we revived these samples and measured the growth of genotypes from each line on every individual substrate. Lines were founded from clones, so that all variance expressed by evolved genotypes originated through novel mutations during the selection experiment. The overall goal of our work was to test the main prediction of the ecological theory of adaptive diversification: diversity should evolve at a greater rate in environments with more ecological opportunity, and this diversification will occur as a result of selection for specialization on different resources in order to escape competition.

Materials and Methods

Ancestral Strain

We used clonal isolates of *Pseudomonas* strains SBW25 and SBW25ΔpanB to found eight replicates of five selection lines. SBW25ΔpanB is an isogenic strain of SBW25 containing a complete deletion of the panB gene. The panB gene is used to synthesize the vitamin pantothenate and when plated on indicator plates with a low concentration of pantothenate (2.4×10^{-6}%), SBW25ΔpanB grows noticeably smaller colonies than SBW25. This marker is selectively neutral and has no effect when pantothenate is present in high concentrations (Rainey 1999; MacLean et al. 2005). The two strains were mixed in roughly equal proportions to form a common pool to start the experimental populations. The ancestral clones were kept frozen at −80°C during the experiment in a mixture of 50% glycerol:50% water (v:v).

Selection Experiment

We chose four carbon substrates involved in different pathways important for *Pseudomonas* metabolism: glucose, fructose, serine, and succinic acid. Two replicate lines were selected on each of the four carbon substrates, and eight replicate lines were selected on a mixture of all four substrates. Single-substrate environments will hereafter be referred to as ''simple'' environments and the four-substrate environment will be referred to as a ''complex'' environment. We grew populations on 96-well plates with each well containing an M9 salt solution (NH_4Cl 1 g/L, Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L) supplemented with a high concentration of pantothenate (2.4×10^{-3}%) and a source of carbon. We maintained a constant concentration for each substrate (0.3 g/L per substrate), rather than maintaining a constant total concentration for each environment. This decision was made so that the rewards of specializing on a specific substrate were equal in simple and complex environments (Barrett et al. 2005). A consequence of this design is that productivity increases with complexity. Every 24 h we transferred selection lines by using a 96-pin replicator to ''print'' the populations grown on a selection plate onto a fresh selection plate. The replicator transfers 0.06–0.07 μl of culture ($\sim 1 \times 10^5$ cells) on each pin to give a dilution factor of approximately 3000-fold per transfer. We continued the selection experiment for 80 transfers, which is equal to about 900 generations. Every ten transfers (~100 generations), we froze all of our lines at −80°C in a mixture of 50% glycerol:50% water (v:v).

Assay

Before our assay, we reconditioned cultures in 96-well microplates containing dilute M9KB medium (NH_4Cl 0.1 g/L, Na_2HPO_4 0.6 g/L, KH_2PO_4 0.3 g/L, NaCl 0.05 g/L, glycerol 1g/L, protease peptone 2 g/L) at 28°C for 24 h. We serially diluted and plated out cultures from each replicate line on solid agar plates. We then randomly picked eight colonies from each line and grew these isolates in dilute M9KB medium at 28°C for two days. We diluted and starved cultures in M9 salt solution for at least two hours before the assay began. We then added 20 μl of starved cells (1×10^6 viable cells) from each culture to 96-well plates. Each well on each plate contained 180 μl M9 solution plus one of the carbon substrates used in the selection experiment. We scored two replicates of each isolate on every assay substrate (2 replicates × 8 isolates × 16 lines × 4 assay substrates × 9 time points).

We measured optical density at 660 nm using a Synergy HT narrow beam plate reader (Biotek Instruments, Winooski, VT) at 24 h ± 30 min of incubation at 28°C, so that the assay conditions were identical to those experienced by each population during one transfer in the selection experiment. The optical density score of any given well reflects the scattering of light by bacterial cells, and can therefore be used to measure the growth achieved by the populations. We corrected optical densities by subtracting control well scores from each absolute score. The growth of each line was calculated as the mean of all measurements of isolates from that line. The

response to selection was calculated as the growth of a line minus the growth of the ancestor on the same substrate.

Statistical Analysis

The effect of environmental complexity on adaptation and divergence has been difficult to address because of limitations in the ability to make causal associations between genetic variation and selectively important environmental factors. By measuring genetic variation in growth on substrates present in selection environments, it is possible to determine the ecological consequences of evolved diversity. We analyzed the variance in growth within populations by partitioning into genetic, environmental, and genotype-by-environment (G×E) interaction components. We used the genetic variance component to estimate the diversity within each population. To follow the changes in diversity through time, we conducted analysis of covariance (ANCOVA) with JMP 4.0 software (SAS Institute, Cary, NC). We determined the rate at which new genetic variance evolved per generation, V_m, as the slope of the regression of genetic variance versus generation (Goho and Bell 2000). To standardize this ''mutational heritability'' so that it was directly related to the response to selection, we divided by the standing environmental variance, V_E, which we estimated from the y-intercept of the fitted linear regression (Lynch 1988). Note that this is based on the assumption that, with a genetically uniform ancestral population, the phenotypic variation is equal to the environmental variation at the start of the experiment. Thus, this value is not equivalent to the environmental variance component determined through partitioning of the variance in growth among substrates. The standardized mutational heritability, V_m/V_E, can be used to compare the rate of accumulation of genetic variance for different traits or different organisms.

If genetic variance within populations reflects adaptive diversification driven by competition for resources, sympatric genotypes should exhibit differentiation in phenotypic characters related to resource consumption (Schluter 2000). We estimated the phenotypic similarity between a pair of evolved genotypes at about generation 900 as the Euclidean distance in a multidimensional space in which each substrate represents one dimension. The Euclidean method estimates the distance between two genotypes, A and B, as $d(\mathrm{A}, \mathrm{B}) = (\Sigma_i (x_i - y_i)^2)^{1/2}$, where x_i and y_i are the mean scores of genotypes A and B, respectively, on substrate i, and the summation is over all substrates. We calculated the score of a single genotype in a given environment as the mean of the two replicate measurements. We used the distance matrix of all genotypes to calculate the average distance between genotypes from either the same or different lines.

RESULTS

Adaptive Diversification through Time

The growth of lines increased nonlinearly with the number of generations under selection (Fig. 1A). By the end of the experiment, the mean growth was 0.023 (SE = 0.0006), representing an increase of 90% over the ancestor. The response to selection varied among lines (SD = 0.003), with an observed range of 0.007 to 0.015. In contrast to the mean

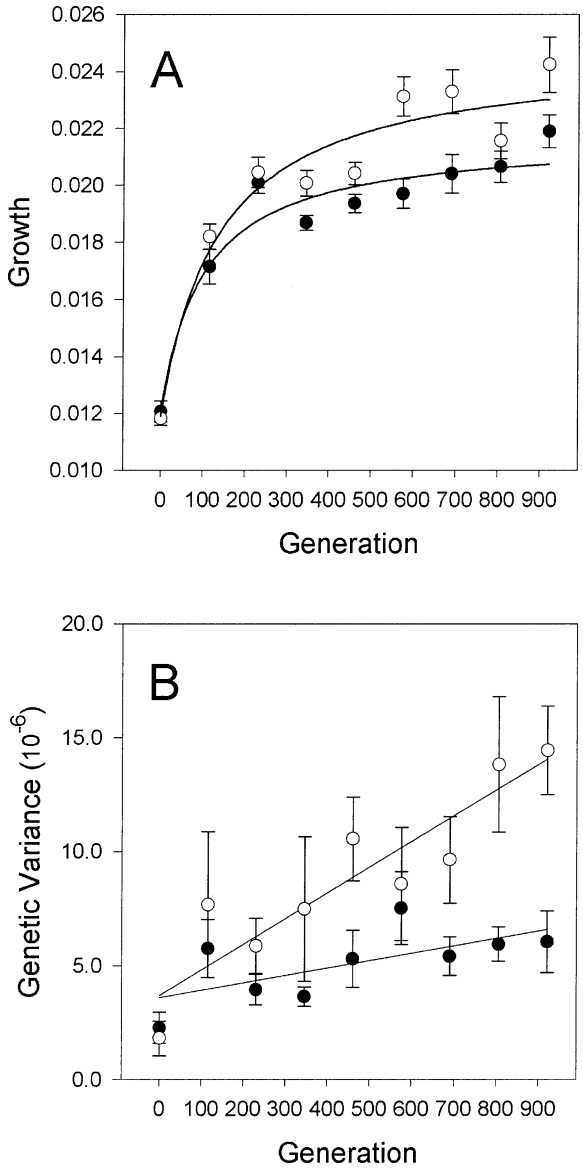

FIG. 1. (A) Adaptation in *Pseudomonas fluorescens* through time. The growth of lines cultured in simple (filled circles) and complex (open circles) environments is plotted against generation. Growth is calculated as the mean corrected growth of all simple- or complex-selected lines across all carbon substrates. Growth increased with number of generations under selection ($F = 180.98$, df = 1, $P < 0.0001$). Lines selected in complex environments had greater average growth than lines selected in simple environments ($F = 14.58$, df = 1, $P < 0.005$). The solid lines show the best fit of a hyperbolic model to the data. Error bars show ±1 SE. (B) The evolution of genetic variance within *P. fluorescens* populations through time. The genetic variance of lines cultured in simple (filled circles) and complex (open circles) environments is plotted against generation. Genetic variance is calculated as the mean genetic variance component within all simple- or complex-selected lines. Genetic variance increased with number of generations under selection ($F = 26.78$, df = 1, $P < 0.0001$). Lines selected in complex environments had greater genetic variance than lines selected in simple environments ($F = 20.59$, df = 1, $P < 0.0001$). Error bars show ±1 SE.

TABLE 1. Analysis of covariance for genetic variance testing effects of time and complexity. We calculated genetic variance by partitioning the growth variance in each line into genetic, environmental, and genotype-by-environment interaction components. We obtained estimates of genetic variance for each level of complexity every 100 generations. The genetic variance estimate for each level of complexity is determined as the average genetic variance of all simple or complex selection lines.

Source	df	MS ($\times 10^{-10}$)	F	P
Time	1	6.69	26.78	<0.0001
Complexity	1	5.14	20.59	<0.0001
Time × complexity	1	2.02	8.08	0.005
Replicate	140	0.25		

growth, the genetic variance within lines showed a linear increase with the number of generations under selection (Fig. 1B). The rate of this increase is different in simple and complex environments, as is indicated by the significant time × complexity interaction in the analysis of genetic covariance (Table 1). After 900 generations of selection, estimates of the genetic variance component are significantly greater for lines selected in complex media than for those selected in simple media (Fig. 2; $t = 3.54$, df = 14, $P = 0.003$). The slopes of the regression for genetic variance versus generation (V_m) were 3.27×10^{-9} (SE 1.14×10^{-9}) for lines selected in simple environments and 1.12×10^{-8} (SE 1.94×10^{-9}) for lines selected in complex environments. The y-intercepts of this regression (V_E) were 3.60×10^{-6} (SE 7.72×10^{-7}) and 3.71×10^{-6} (SE 1.06×10^{-6}) for simple- and complex-selected lines respectively. These estimates of V_m and V_E give mutational heritabilities of $V_m/V_E = 1.0 \times 10^{-3}$ per generation for simple-selected lines and 3.0×10^{-3} per generation for complex-selected lines.

Character Displacement

We used Euclidean distances to determine the differences in metabolic phenotype between genotypes. Relative to simple environments, genotypes selected in complex environments had more within-line phenotypic distance than between-line phenotypic distance ($\chi^2_{0.05,1} = 29.80$, $P < 0.0001$). Thus, complex-selected genotypes from different lines often share a common phenotype, but sympatric complex-selected genotypes show divergent phenotypes. To determine whether the genetic diversity in each complex-selected population would fit into distinct phenotypic classes we performed *t*-tests for multiple comparisons on each line (Travisano et al. 1995). The eight genotypes assayed in each line were treated as fixed, and we applied a sequential Bonferroni correction since eight *t*-tests were performed (Rice 1989). A further Bonferroni correction was incorporated because we performed four such tests (one for each substrate), so that the experiment-wise type I error rate for each substrate was 0.05/4 = 0.0125. One or more genotypes were placed in a separate phenotypic class if they differed from all other genotypes in their performance on at least one substrate (Travisano and Lenski 1996). Using this method, the average number of phenotypic classes within each population was very low (mean = 1.38), suggesting that most genotypes from the same population share a common phenotype. This is because in most populations the performance of genotypes on each substrate is characterized by a fairly continuous distribution. There are few examples of specialists that are significantly better than all other genotypes on a specific substrate. There is, however, substantially greater G×E interaction variance in complex-selected populations than in simple-selected populations (two-tailed $t = 7.80$, df = 14, $P < 0.001$). Moreover, the majority of G×E interaction variance is due to inconsistency as opposed to responsiveness (average fraction of G×E attributable to inconsistency in complex lines = 86%). Thus, differences between sympatric genotypes from complex environments are not due to large differences in performance on each substrate but rather because the rankings of the genotypes vary on different substrates (Barrett et al. 2005).

DISCUSSION

Theory predicts that competition for shared resources in a genetically uniform population generates divergent selection for adaptation to alternate resources (Maynard Smith 1966; Geritz et al. 1998; Dieckmann and Doebeli 1999; Doebeli and Dieckmann 2000). A clear prediction of this theory is that diversity will increase with the number of resources available in the environment. There is evidence from natural systems to support this prediction (Murdoch et al. 1972; McKane et al. 2002; Armbrecht et al. 2004). However, these studies have been conducted on ecological timescales, in the sense that the organisms involved retain their properties unchanged during the experiment and no diversity evolves de novo. Microbial model systems have provided a way to follow replicated adaptive diversification over evolutionary timescales. MacLean et al. (2005) showed that selecting *P. fluorescens* in complex mixtures of undefined substrates results in the evolution of communities of genotypes specialized to consume different resources. However, the performance of these genotypes was assayed on different substrates than were present in the selection environments, making it difficult to assess the ecological consequences of evolved diversity. Here, we followed the evolution of diversity under chemically defined selection conditions and assayed performance on the same substrates that were present in the selection environments. In addition, we provided a control for the effect of complexity by also selecting in simple environments consisting of only a single resource.

Even in simple environments it appears that some diversity can evolve and be maintained. Although this was not predicted, it has been shown in previous experiments. For example, Rosenweig et al. (1994) demonstrated that a single *E. coli* genotype selected in a glucose-limited environment partitions the primary resource through its own metabolic activities. Excreted metabolites provide ecological opportunity for mutants with an enhanced ability to grow on these metabolites. When niche specialist mutants arise, they are maintained through the operation of density-dependent processes (Rainey et al. 2000). Other studies have also shown the emergence of diversity from genetically uniform populations during the course of evolution in simple environments (Turner et al. 1996; Rozen and Lenski 2000). It is possible that similar resource partitioning occurred in the simple environments in our experiment. Theory predicts, however, that cross-feeding

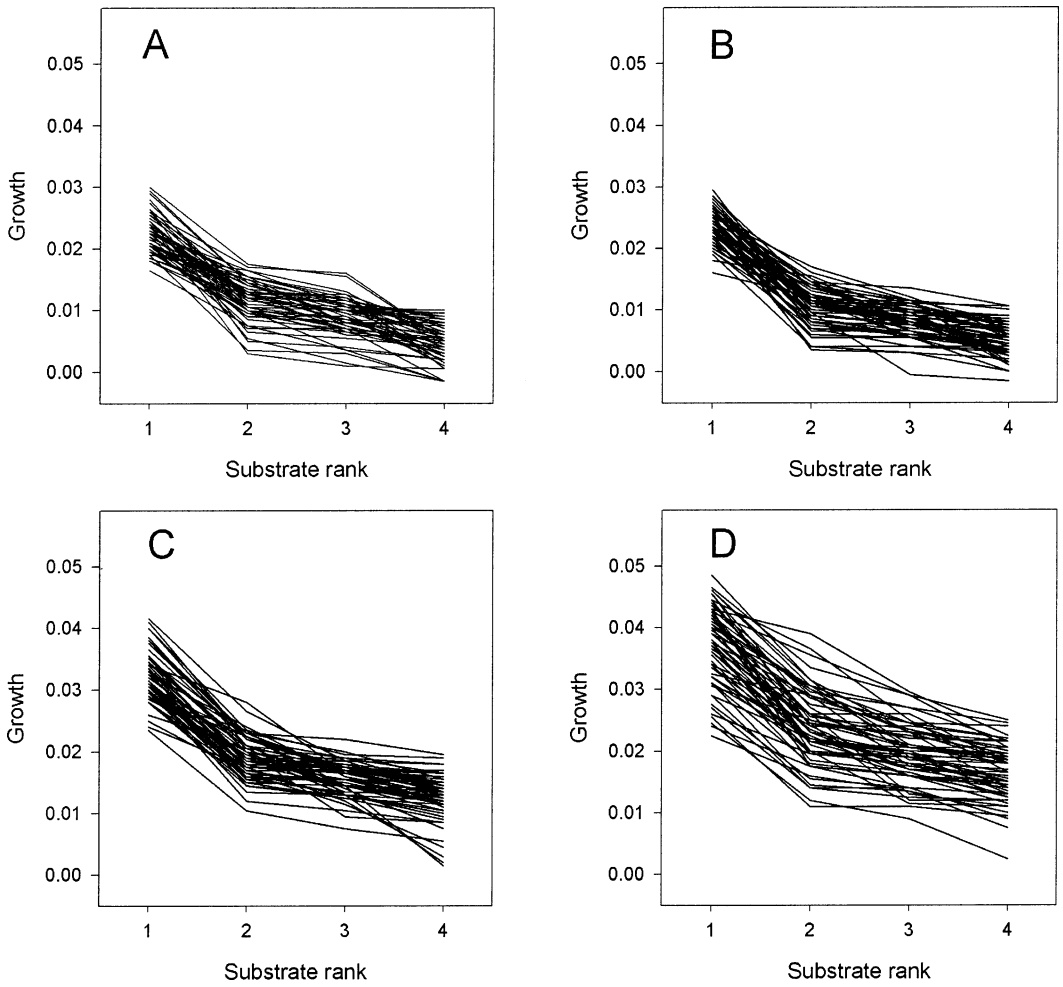

FIG. 2. Ranked growth curves for experimental data. Substrates are ranked by the growth of each genotype, such that the substrate at each rank may be different for different genotypes. The slope of the curves reflects environmental variance, the width of a band of curves reflects genetic variance among genotypes. Upper panels show the growth of (A) simple-selected and (B) complex-selected genotypes on each of the four substrates in the assay at generation zero. There was no significant difference between the mean ($t = -0.68$, df = 126, $P = 0.50$) or within-line variance ($t = -0.45$, df = 14, $P = 0.66$) of simple- and complex-selected genotypes. Lower panels show the growth of (C) simple-selected and (D) complex-selected genotypes on each of the four substrates in the assay at generation 900. Complex-selected genotypes had higher mean ($t = 3.36$, df = 126, $P = 0.001$) and within-line variance ($t = 3.36$, df = 14, $P = 0.003$) than simple-selected genotypes.

is unlikely to evolve when using a transfer protocol like ours (Doebeli 2002). Although some genetic variance evolved in simple environments, the amount was modest relative to the complex environments. Thus, evolution in simple environments can be characterized as a gradual turnover of progressively better-adapted genotypes. In contrast, in complex environments, the ancestor diversifies into a community of metabolically differentiated genotypes. Genotypes that evolved in the same populations exhibit more phenotypic differentiation from each other than to genotypes that evolved in different populations. This pattern is consistent with ecological character displacement, in which evolutionary shifts of phenotypic traits occur in response to competition for resources between similar genotypes.

The values that we obtained for V_m/V_E, 1×10^{-3} and 3×10^{-3} per generation, are comparable with other studies investigating the accumulation of genetic variance through time (reviewed in Houle et al. 1996). In the study with the population sizes, generation times and culture conditions closest to ours, the estimate is approximately 4.5×10^{-3} (Goho and Bell 2000). This is considerably greater than the average value obtained in our experiment, suggesting that our system has accumulated either less genetic variance or expressed greater environmental variance. However, their study may give biased estimates of V_m, because the assay conditions were different from the selection environment. It has been argued that the genetic correlation is a declining function of environmental variance, and since our selection experiment and assay were conducted in the same conditions, our estimate of V_m can be expected to be reliable (Bell 1992). Although there is some variation in V_m/V_E among studies, the overall impression is that values are remarkably consistent. Despite the various methods used to score diverse characters on organisms including *Drosophila*, *Tribolium*, *Daphnia*, *Caenorhabditis*, *Chlamydomonas*, mice, and several crop plants, almost all V_m/V_E values are between 10^{-4} and $5 \times$

10^{-2}, and the majority are roughly equal to 10^{-3} (Houle et al. 1996; Lynch 1988). This suggests the intriguing possibility that there may be general rules governing the accumulation of genetic variance in evolving populations. Our experiment is the first to follow the evolution of diversity over a long period of time in environments that vary in the amount of ecological opportunity available. Complex environments provided three additional substrates over the single substrate present in the simple environments. Interestingly, the V_m/V_E in complex environments was exactly three times that found in simple environments, implying that the rate of genetic accumulation may be roughly proportional to the amount of ecological opportunity present. An alternative explanation for our results is that diversification is not driven by ecological opportunity, but rather by productivity. In this case, diversification still occurs through niche specialization, but it proceeds more quickly in highly productive environments. Further tests at other levels of ecological complexity and with controls for productivity will be required to test the relationship between complexity and diversity rigorously.

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