Drift Increases the Advantage of Sex in RNA Bacteriophage Φ6

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ABSTRACT

The pervasiveness of sex and recombination remains one of the most enigmatic problems in evolutionary biology. According to many theoretical models, recombination can increase the rate of adaptation by restoring genetic variation. However, the potential for genetic drift to generate conditions that produce this outcome has yet to be studied experimentally. We have designed and performed an experiment that reveals the effects of drift on existing genetic variation by minimizing the influence of variation on beneficial mutation rate. Our experiment was conducted in populations of RNA bacteriophage Φ6 initiated from a common source population at varying bottleneck sizes. The segmented genome of this virus results in genetic exchange between viruses that co-infect the same host cell. In response to selection for growth in a high-temperature environment, sexual lines outperformed their asexual counterparts on average. The advantage of sex attenuated with increasing effective population size, implying that the rate of adaptation was limited by clonal interference among segments caused by drift. This is the first empirical evidence that the advantage of sex during adaptation increases with the intensity of drift.

ALMOST every organism has some form of genetic exchange among individuals (Bell 1982; Awadalla 2003). As an evolutionary strategy it is quite counterintuitive that a successful individual would risk shuffling its genes with another, having no guarantee of improving its genetic standing; nevertheless, an abundance of theoretical models have been developed that describe conditions favoring sex and genetic recombination (Otto and Lenormand 2002). Experimental studies are essential for discriminating among these models, and yet there have been relatively few attempts to test them (Rice 2002). A common theme shared among these models is that genetic recombination becomes selectively favorable by eliminating maladaptive linkage disequilibria (Otto and Lenormand 2002). It remains to determine only whether these linkage disequilibria are being generated by epistatic selection (Eshel and Feldman 1970) or genetic drift (Fisher 1930; Muller 1932). Although there are many potential sources of epistasis, such as metabolic flux (Szathmary 1993) or ecological competition (Peck and Waxman 2000), only a narrow range of epistatic interactions will sustain an advantage of recombination (Barton 1995). Of the epistatic interactions measured in empirical studies, too few fall within this range for epistasis alone to explain sufficiently the ubiquity of recombination (Rice 2002). On the other hand, all populations are finite, and genetic drift is inevitable. Indeed, recent theoretical work has shown that drift will also generate conditions favoring recombination during adaptation (Otto and Barton 2001).

Genetic drift creates linkage disequilibrium by chance, such that random variation in genotype frequencies accumulates over time. Unlike epistasis, drift is not predisposed toward reinforcing particular genotypes, and conditions that favor recombination are created as frequently as those that oppose it. Nonetheless, a net advantage of recombination emerges when selection acts on newly generated linkage disequilibria, because the rate of response to selection varies with the type of linkage disequilibrium (Hill and Robertson 1966). When beneficial alleles are found together more often than expected (defined as positive linkage disequilibrium), the genotypes that are the most dissimilar in fitness become the most abundant; this asymmetry leads to the rapid fixation of alleles (Figure 1). Although recombination is penalized initially by dispersing the fittest genotype, this effect attenuates as beneficial alleles rapidly approach fixation. Conversely, when different beneficial alleles tend to be in separate individuals (negative linkage disequilibrium), the most abundant genotypes are more similar in fitness. Selection becomes less efficient at increasing the frequency of individual alleles, and negative linkage disequilibria consequently become ensnared in the population. This outcome is known as the Hill-Robertson effect (Hill and Robertson 1966) or clonal interference (Muller 1964). By regenerating the fittest genotype, recombination increases genetic variation in fitness and hence the rate of response to selection (Figure 1).

Because the intensity of genetic drift decreases with increasing effective population size, we should expect that the advantage of recombination is greater in

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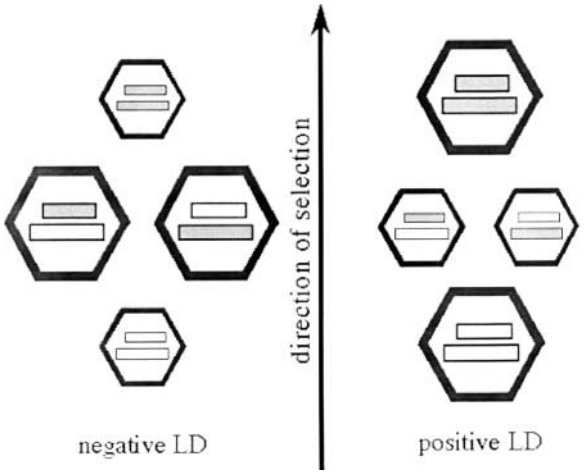

Figure 1.—A cartoon depiction of the Hill-Robertson effect. Shown here are two equally probable bottleneck samples from a source population of viruses having two segments of two types (shaded, open) at equal frequency and no linkage disequilibria. Shaded segments are favored by selection. The relative size of the viruses represents random departures of genotype frequencies, producing linkage disequilibria (LD). Negative LD causes most of the genetic variation to become orthogonal to the direction of selection. As a result, the response to selection is below the genetic potential of the population. Conversely, under positive LD the genetic variance is mostly aligned with the direction of selection and the response to selection is rapid. Over time, the linkage disequilibria that remain will be increasingly negative.

smaller populations or in populations that have recently undergone a severe bottleneck (Otto and Barton 2001). However, when populations are very small, it becomes unlikely that unfixed beneficial alleles are present simultaneously at two or more sites, which is a prerequisite for recombination to have any influence on adaptation (Fisher 1930). To reveal the effect of drift in small populations, we have conducted an experiment designed so that adaptation is not limited by the beneficial mutation rate. First, our experimental populations were descended from a genetically diverse source population that was assembled with a known frequency of beneficial alleles. Second, different bottleneck sizes (10^2, 10^3, and 10^4) were applied only once to produce drift in our populations, which were all maintained thereafter at identical high densities to keep mutation rates uniform while retaining the effects of drift. This experimental design allowed us to obtain the first empirical evidence that the advantage of recombination during adaptation increases with increasing genetic drift.

Our experiment was conducted in populations of the RNA bacteriophage Φ6. The Φ6 genome consists of three segments, and although homologous recombination within segments is very rare, they are freely exchanged between viruses infecting the same cell (*e.g.*, reassortment; Horiuchi 1975). Hence, there are effectively three loci in the Φ6 genome. Genetic recombination has been recognized increasingly as an important force underlying rapid evolution in viruses (Chao 1992; Worobey and Holmes 1999). Although our result can be generalized to any organism, it is particularly relevant to the evolution of pathogenic viruses that regularly experience severe bottlenecks during transmission between hosts (Bergstrom *et al.* 1999).

MATERIALS AND METHODS

Growth and plating conditions: Our standard bacterial host for growing the bacteriophage Φ6 is *Pseudomonas syringae sv. phaseolicola.* Cultures of *P. phaseolicola* were grown in LC media (modified Luria broth; Mindich *et al.* 1976) inoculated with single colonies from a frozen stock and grown to stationary phase overnight at 25°. For growth on LC plates (*e.g.*, serial transfer), populations of Φ6 were combined with *Pseudomonas phaseolicola* in LC soft agar and incubated for 24 hr. Approximately five generations occur within this time interval. Unless indicated otherwise, experimental lines were always grown in the selective high-temperature environment (33°). The density of phage after growth was estimated by plating a known dilution at 25°, an optimal temperature at which Φ6 will form large plaques irrespective of prior temperature adaptation. For growth from high initial densities of phage (10^4), large plates (150-mm diameter) were used to prevent plaques from overlapping. The separation of plaques was necessary to restrict uncontrolled recombination among phage during selection. Although co-infection within plaques was unavoidable, each plaque is effectively a clonal population and only recombination among phage from different plaques would have affected linkage disequilibria. Plating conditions (*e.g.*, soft agar volume, bacterial density) for large plates were adjusted to be identical to standard plates (100-mm diameter) that were used for growth from low initial densities ($\leq 10^3$ phage). Further details on growth, handling, and frozen storage of bacteria and phage are available in previous work (Chao *et al.* 1992).

Construction of the source population: Our experimental source population was initialized from a mixture of a high-temperature (33°) adapted clonal population with its ancestor at a respective ratio of 1:9. Hence, the initial frequency of alleles beneficial in the high-temperature environment was ~0.1. The ancestor was a 25°-adapted population of Φ6 (DH25) that has been serially transferred for nearly 1000 generations at a large effective population size ($N_e \geq 10^4$). By selection at a higher temperature (33°), a 33°-adapted line (DH33) was derived from DH25. After 50 generations of selection, the growth rate of DH33 increased more than twofold relative to DH25 at 33°. A single plaque was isolated from DH33 and used in construction of the source population. A host-range mutant (DH33*h*) that exhibits growth on an alternate host, *Pseudomonas pseudocaligenes ERA*, was also isolated from DH33. This marked genotype is useful for measuring fitness by competitive growth as described below. The mutation responsible for this range expansion is located on the medium-length genome segment. Using this genetic marker, the medium segment from DH33*h* was introgressed into the DH25 background after five backcrosses to determine that alleles conferring higher fitness at 33° were located on at least two of three genome segments (D. Hong, unpublished data). These crosses were accomplished by combining equal amounts of DH33*h* and DH25 phage into a high-concentration mixture (10^8 phage/ml) and transferring a 10-μl drop onto a layer of soft agar inoculated with *P. phaseolicola.* A high rate of co-infection, and thereby recombination among phage, was ensured by this spot-plating procedure.

To disperse the linkage disequilibria caused by the mixture

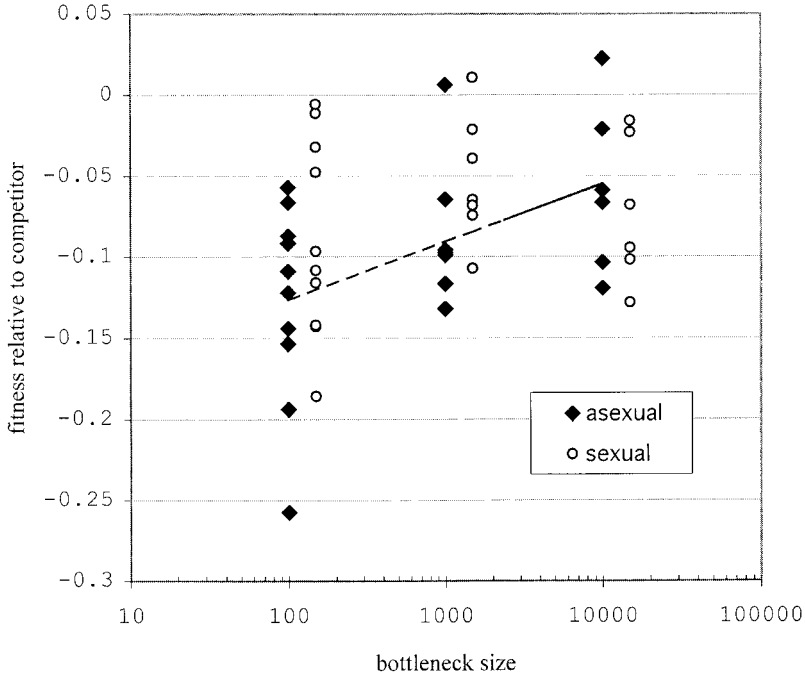

FIGURE 2.—Asexual fitness of bottlenecked lines. The response to selection following a single bottleneck is diminished in asexual lines (solid diamonds) descended from smaller bottleneck sizes. The dashed line represents a least-squares linear fit to the asexual fitness values that illustrates this trend ($F = 6.82$, $P = 0.016$). This implies that less genetic variation was available to selection in these lines. Fitness values of sexual treatments (open circles) are also shown for comparison; they are displaced to the right for clarity, but there is no actual difference between sexual and asexual treatments in bottleneck size. Each point represents the average of six replicate estimates of fitness from assays of competitive growth relative to the DH33*h* competitor.

of the two genotypes, we transferred the source population five times by spot plating so that at least five rounds of recombination occurred between genome segments. These transfers were performed at 29°, at which temperature the DH25 and DH33 genotypes are similar in fitness (D. HONG, unpublished data), to prevent change in initial allele frequencies.

Bottlenecking, selection, and recombination: We initiated 24 experimental lines by sampling from the source population at three bottleneck sizes: 10 replicate lines from a sample of 10^2 phage, 8 from 10^3 phage, and 6 from 10^4. Because the source population had undergone several rounds of recombination, any linkage disequilibria in these lines was the direct result of genetic drift caused by these bottleneck treatments. All lines were subsequently transferred at a large size (10^4) for 10 generations to select for growth at high temperature. This should have allowed selection to increase the frequencies of beneficial alleles at varying rates, dependent on the type of linkage disequilibria created by drift (HILL and ROBERTSON 1966). After selection, each line was split into sexual and asexual treatments. These treatments were carried out in liquid cultures consisting of a known concentration (5×10^8 cells/ml) of log-phase *P. phaseolicola*, as determined by adsorption of 600 nm light in a spectrophotometer. In sexual treatments, the density of phage added to the log-phase culture was adjusted so that there were on average 4 phage per cell. In contrast, there were 4×10^{-4} phage per cell on average in asexual treatments. All the infected cultures were incubated at 25°, at which temperature the rate of adsorption is maximized, until sufficient time had elapsed for ~90% of the phage to have adsorbed to cells. Each culture was then centrifuged to remove unadsorbed phage, and the pellet of infected cells was transferred into fresh media to allow cells to burst and release phage progeny. The cultures were then immediately filtered and frozen.

Measuring the advantage of sex by competition assay: Sexual and asexual treatments from all lines were each reinitialized from frozen stocks by a single transfer of 10^4 phage onto large plates at 33°. One 10^3-bottleneck replicate was lost during recovery, although there was no prior indication that the line was genetically low in fitness. The fitness of each treatment was assayed by competitive growth against the DH33*h* host-range mutant. When DH33*h* is grown on a mixed lawn of *P. phaseolicola* and *ERA*, it infects both hosts and forms clear plaques. Conversely, nonmutant phage will infect only *P. phaseolicola*, and cloudy plaques are formed in mixed lawns when *ERA* is plated at a dilute concentration. As a result, the densities of both competitors can be estimated simultaneously on the same plate to control for environmental variation. To estimate relative fitness, we compared the initial ratio (R_0) of nonmutant phage and DH33*h* densities to the final density ratio (R_1) after selection at 33°. Each experimental population was mixed with an equal number of the DH33*h* competitor, and the realized R_0 was estimated from three replicate plates on a mixed lawn at 25°. From each mixture, six replicates each of ~10^3 phage were plated with *P. phaseolicola* and incubated at 33° for 24 hr, such that the competitive environment matched the environment of selection. Thus, a total of 10 generations of high-temperature selection occurred after the separation of bottlenecked lines into sexual and asexual treatments. Offspring phage were obtained from all 276 plates, serially diluted in 96-well plates, and replated on a mixed lawn at 25° for density estimates. Estimates of R_1 were obtained by averaging the density ratios measured from each of the six replicates.

Estimates of the relative fitness of phage were obtained from the formula $W = R_1/R_0$. We used the log transformation of this ratio, which is proportional to the relative Malthusian fitness, for the statistical analyses conducted in the JMP statistical package for Macintosh. To evaluate the effect of the effective population size on the advantage of sex, we used a linear regression of the difference in log W between sexual and asexual treatments against the log-transformed bottleneck size. We used the log transformation of bottleneck size because the intensity of drift varies reciprocally with the effective population size.

A computer simulation that mimicked the evolution of our experimental populations was written in C and employed to assist the interpretation of our results. Bottlenecked populations were generated by iterative binomial sampling, assuming initial allele frequencies of $P = 0.1$ at two or three loci. Subsequent selection and recombination altered the genotype

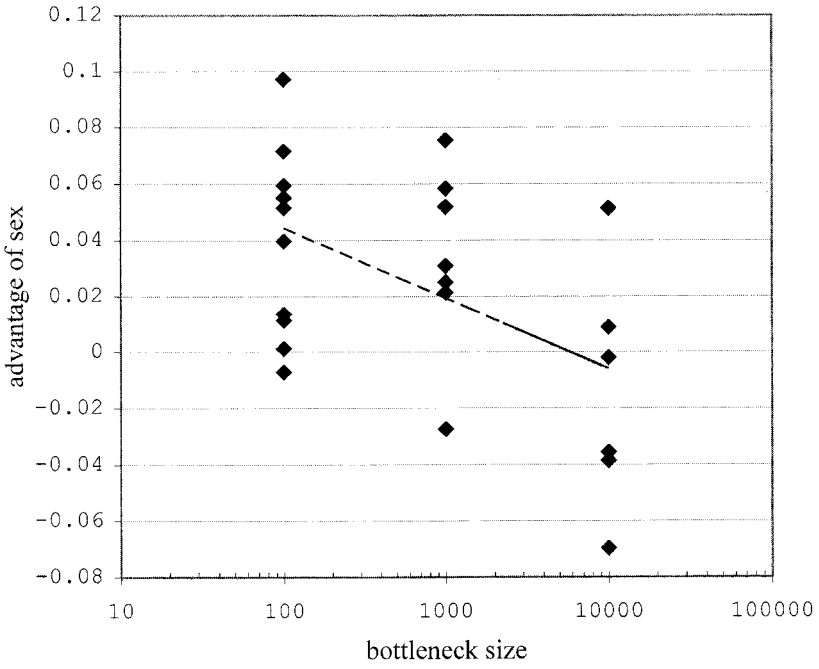

FIGURE 3.—The advantage of sex decreases with increasing bottleneck size. Advantage of sex is quantified by the difference between sexual and asexual estimates of fitness relative to the DH33 competitor, as determined by the difference in log-transformed density ratios before and after competitive growth (see MATERIALS AND METHODS). For all bottleneck-size treatments, the sexual lines on average were of greater or equal fitness to their corresponding asexual lines. Each point represents the line average over six replicate competitive growth assays. The dashed line represents a least-squares linear fit to the data ($F = 7.22$, $P = 0.014$). The variance among lines within treatments is significant (nested ANOVA: $F = 3.73$, $P < 0.0001$), but there is no substantially greater variance in any particular treatment.

frequencies according to deterministic recursion equations (KIMURA 1965a). Fitness effects of individual loci were assumed to accumulate multiplicatively. The effect of sex was evaluated by the change in variance of fitness immediately before and after recombination.

RESULTS AND DISCUSSION

Severe bottlenecks impede response to selection: In bottlenecked lines descended from a common source population, the rate of response to selection for growth at high temperature was determined by measuring the fitness of the bottlenecked lines relative to a marked competitor, DH33*h*. By excluding recombination in the asexual treatments within the bottlenecked lines, we measured the effect of bottleneck size on the response to selection. The relative fitness of asexual treatments at 33° increases significantly with increasing bottleneck size (Figure 2; $F = 6.82$, $P = 0.016$). This implies that less genetic variance was available for selection in populations of smaller effective size. Because we restricted variation in mutation rate among populations, it is unlikely that this effect was due to the accumulation of novel beneficial mutations in populations with large effective sizes. It is also unlikely that lines descended from smaller bottlenecks failed to sample beneficial alleles from the original source population, because those alleles were provided at a substantial frequency ($P_0 = 0.1$). Finally, although we cannot rule out the possibility that deleterious alleles present in the source population became fixed in smaller bottlenecks, it is unlikely to have occurred so rapidly. Accordingly, the most probable explanations for this result are that negative linkage disequilibria created by drift in small bottlenecks persisted in these populations and that subsequent clonal interference limited the response to selection on average.

Greater advantage of sex with smaller bottlenecks: The overall fitness of sexual treatments averaged across all bottleneck sizes was significantly greater than the average asexual fitness (Student's one-tailed *t*, $P = 0.006$). However, sexual and asexual treatments in the six lines descended from a bottleneck of 10^4 phage were not significantly different in fitness (Student's one-tailed *t*, $P > 0.1$) and did not contribute to this result. At this large bottleneck size, drift should be weak given the high initial allele frequencies, and linkage disequilibrium would be generated mostly by epistatic selection. Introgressing the medium-length segment of DH33*h* into the DH25 background determined that the contribution of that segment was additive with the remaining segments (D. HONG, unpublished data). Furthermore, in Φ6 genes located on the same genome segment tend to share similar functions (MINDICH 1999). Hence, we should expect that the capacity for epistasis to generate conditions favoring sex would be limited in this system. This is consistent with the lack of divergence in fitness between sexual and asexual treatments in the lines with the largest effective population size.

The most important result obtained in this experiment is that the advantage of sex decreases with increasing bottleneck size (Figure 3; $F = 7.22$, $P = 0.014$). Because the intensity of drift is inversely proportional to the effective population size, this result implies that the advantage of sex during adaptation was driven by genetic drift. This is consistent with empirical evidence (CHAO *et al.* 1992, 1997) that sex can also become advantageous because of Muller's ratchet, the stochastic accumulation of deleterious mutations that is fundamentally

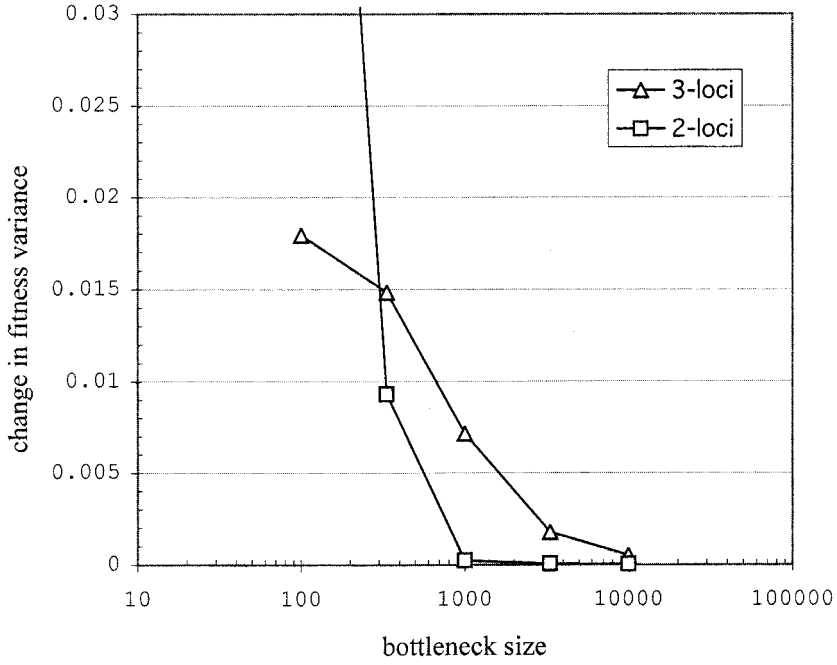

FIGURE 4.—Simulations favor a three-locus model of selection. Each point represents the average of 10,000 populations generated by pseudorandom sampling from a source population with two or three loci polymorphic for beneficial alleles at an initial frequency of 0.1. The fitness effects of alleles were adjusted so that the fittest genotype had the same advantage over the wild type in either case. Variance in fitness was calculated directly from genotype frequencies before and after recombination. The difference represents the effect of recombination on the rate of response to selection. We truncated the graph so that the divergence between two- and three-locus models could be clearly illustrated. When two loci are under selection, there is a large advantage of sex at $N_e = 100$ (increasing variance by 0.077) that rapidly diminishes to zero with increasing bottleneck size. In contrast, the three-locus model predicts that the advantage of sex is sustained for larger bottleneck sizes, which is more consistent with our experimental results.

based on the same process (MULLER 1964). On the other hand, the reduction of the mutation load would not be as effective as strong positive selection at promoting recombination. For example, increases in recombination rates have been frequently observed after periods of artificial selection on phenotypes with no obvious association with recombination (reviewed in OTTO and BARTON 2001).

This result might also have been caused by an increasing recombination load (BARTON 1995) in populations of larger effective size, if the source population was polymorphic for rare alleles with strong epistatic interactions. Immediately following recombination, a loss of fitness would be caused by the breakdown of linkage disequilibria accumulated by epistatic selection. It is unlikely, however, that such rare interactions could cause a recombination load so substantial that its effects would be sustained over 10 generations of selection. The contribution from DH33 to the experimental source population was clonal, and DH25 had been maintained at a high density with frequent recombination for nearly 1000 generations. Hence, any genetic variation causing a recombination load should have been exposed to selection. No substantial loss of fitness was observed during the initial rounds of recombination during the construction of the source population.

Although we have removed the effect of variation in mutation rate from our experiment, it doubtless can constrain the advantage of sex in natural populations (FISHER 1930). This has recently been verified in experimental populations of Chlamydomonas descended from a clonal ancestor (COLEGRAVE 2002). A significant advantage of sex was detected only in populations maintained at a very large size (10^6). For recombination to affect the rate of adaptation, there must be two or more beneficial mutations segregating at different loci in the population, which is increasingly unlikely when small populations must accumulate novel genetic variation. However, substantial genetic variance often exists in populations prior to selection in a novel environment (KIMURA 1965b). This is especially true for organisms with a high mutation rate, such as RNA viruses (DRAKE 1993). Hence, variation in mutation rate likely has less influence on the advantage of sex than does the genetic drift of existing genetic variation. Regardless, the previous result obtained in Chlamydomonas (COLEGRAVE 2002) and our present result are not mutually exclusive. Rather, they represent two processes that act together along a continuum of effective population size, predicting that the greatest advantage of sex occurs at an effective population size that is neither too small nor too large.

Number of loci under selection: Computer simulations that mimic the evolution of our experimental populations were used to evaluate the effect of varying the number of loci under selection. We observed that after 24 hr of growth at 33°, DH33 yields ~200-fold greater phage density than DH25 does. Assuming five generations of growth occurred in this time interval, this implies that the DH33 genotype has an ~2.8-fold increased growth rate. This selective advantage was partitioned among two or three loci, assuming individual allelic contributions are equal and combine multiplicatively. The effect of recombination on genetic variance in fitness is shown for both two- and three-locus cases in Figure 4. In the two-locus case, the advantage of sex is greatest at the smallest bottleneck size (10^2), but diminishes rapidly to zero with increasing bottleneck size. In

contrast, the advantage of sex is sustained over a larger range of bottleneck size in the three-locus case, which is more consistent with our experimental results. A similar trend occurs for the change in mean fitness caused by recombination. This implies that alleles beneficial for growth at high temperatures were present on all three genome segments of the DH33 population. Variation in the number of loci under selection has a strong influence on the advantage of sex because with more selected loci, the genotype with all beneficial alleles becomes increasingly rare and is less likely to be sampled in larger bottlenecks. Subsequently, this genotype can be reintroduced only by recombination.

Implications for viral evolution: Many viruses exhibit homologous recombination (Lai 1992), and many others also reassort genome segments during co-infection (*e.g.*, influenza A; Worobey and Holmes 1999; Steinhauer and Skehel 2002; Awadalla 2003). In the latter case, variation in recombination rate is probably limited by the immediate constraints imposed on genomic structure (but see Onodera *et al.* 1998). Although recombination itself may not be always evolutionarily malleable, its presence has important evolutionary consequences for the fitness of viral pathogens. A pathogenic virus will periodically undergo severe population bottlenecks during transmission between hosts (Bergstrom *et al.* 1999; Awadalla 2003), followed by exponential growth, selection, and recombination. Because of the high mutation rates exhibited by viruses, the source population of infection almost certainly has abundant genetic variation in fitness (Holland *et al.* 1992; Drake 1993). In sum, our experimental design loosely mimics a single infective cycle of a virus after transmission to a new host. Our results suggest that genetic drift caused by frequent narrow bottlenecks in horizontal transmission can create an evolutionary advantage for increased recombination in viral pathogens.

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